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Efficiency of bowel preparation for capsule endoscopy examination: A meta-analysis

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Abstract

Good preparation before endoscopic procedures is essential for successful visualization. The small bowel is difficult to evaluate because of its length and complex configuration. A meta-analysis was conducted of studies comparing small bowel visualization by capsule endoscopy with and without preparation. Medical data bases were searched for all studies investigating the preparation for capsule endoscopy of the small bowel up to July 31, 2007. Studies that scored bowel cleanness and measured gastric and small bowel transit time and rate of cecum visualization were included. The primary endpoint was the quality of bowel visualization. The secondary endpoints were transit times and proportion of examinations that demonstrated the cecum, with and without preparation. Meta-analysis was performed with StatDirect Statistical software, version 2.6.1 (<http://statsdirect.com>). Eight studies met the inclusion criteria. Bowel visualization was scored as "good" in 78% of the examinations performed with preparation and 49% performed without ($P < 0.0001$). There were no significant differences in transit times or in the proportion of examinations that demonstrated the cecum with and without preparation. Capsule endoscopy preparation improves the quality of small bowel visualization, but has no effect on transit times, or demonstration of the cecum.

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Key words: Capsule endoscopy; Bowel preparation; Transit time

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INTRODUCTION

Good preparation before endoscopic procedures is essential for successful visualization. It is also an important factor for patient safety, quality of care, and cost efficiency^[1,2]. The small bowel is difficult to evaluate because of its length and complex configuration. The recently developed Given Diagnostic Imaging System (PillCam, Given Imaging Ltd., Yoqneam, Israel) for evaluation of pathologies of the small bowel^[3-7] can identify lesions that cannot be detected by other techniques^[8,9]. The preparation recommended by the manufacturer is a 12-hour fast after 24-hour intake of clear liquids.

Studies of the effect of bowel preparation for capsule endoscopy on optimal visualization, gastric and small bowel transit times, and rate of cecum demonstration have sometimes reached different conclusions^[10-17]. Sood *et al*^[18], in MRI studies, found no significant difference in transit time between patients prepared with polyethylene glycol or standard methods. In our previous studies, the use of sodium phosphate preparation was not associated with a significant change over fasting in gastric or small bowel transit times, although it yielded a significantly better view of the mucosa^[10,11]. Others reported that both polyethylene glycol and sodium phosphate have a marked accelerating effect on small intestinal transit time^[19,20].

In the present study, we performed a meta-analysis of studies comparing preparation with no preparation before capsule endoscopy of the small bowel. The primary endpoint was the quality of small bowel visualization. The secondary endpoints were gastric and small bowel transit times and proportion of examinations demonstrating the cecum.

FORMULATION OF QUESTIONS

The primary question was: What is the contribution of bowel preparation to successful capsule endoscopy

examination? This raised the subsidiary question of a possible difference in the rates of excellent and good cleaning of the small bowel with and without preparation. In addition, we sought to determine the impact of preparation on gastric transit time, small bowel transit time, and arrival of the capsule to the cecum with the battery still functioning.

CRITERIA FOR CONSIDERING STUDIES FOR THIS META-ANALYSIS

Our study was based on controlled studies of the quality of small bowel capsule examination with and without prior preparation. Two studies of our group were included in the meta-analysis^[10,11]. There was no overlap between the patient populations in these two studies.

SEARCH STRATEGY FOR IDENTIFICATION OF STUDIES

We conducted a bibliographic search of the PubMed (MEDLINE), EMBASE, COCHRANE LIBRARY (Cochrane Database of Systemic Reviews and the Cochrane Controlled Trial Register), CINAHL, and AMED databases up to July 31, 2007 using the following keywords: “capsule endoscopy”, “M2A” (mouth-to-anus), and “PillCam”. The search was directed at English-language medical journals. All papers identified by the electronic database search were examined and additional references were identified from the references listed in each paper.

INCLUSION CRITERIA

Case-control studies investigating the preparation for capsule endoscopy of the small bowel which employed a scoring system for bowel cleanness and measured gastric and small bowel transit time and rate of cecum demonstration were included.

EXCLUSION CRITERIA

Studies that focused on esophageal or colonic examination, did not directly compare preparations, had no control, or did not measure transit times were excluded from the analysis.

STATISTICAL ANALYSIS

For each study, the following variables were extracted and entered into an Excel data sheet: author, journal, year of publication, number of participants, type of preparations, proportion of good bowel visualization, gastric transit time, small bowel transit time, and proportion of cecum demonstration. The meta-analysis was performed with the StatDirect Statistical software, version 2.6.1 (<http://statsdirect.com>). Heterogeneity was checked using χ^2 test (Q-statistics) with significance of $P < 0.05$. With the assumption that the studies are a random sample from a population of studies, we choose to use a random

Table 1 List of 5 case-control studies, comparing proportions of “good scoring” of bowel visualization, fulfilling the inclusion/exclusion criteria ($n = 237$)

Author	Ref.	Preparation	No. with	No. without	“good scoring” with prep.	“good scoring” without prep.
Niv Y, 2004	10	Na-P	22	10	19	3
Viazis N, 2004 ¹	17	PEG 2L	40	40	36	24
Albert J, 2004	12	Simethicone	18	18	14	5
Niv Y, 2005	11	Na-P	23	23	18	12
Ben-Soussan E, 2005	13	PEG 2L	26	16	15	10
Total			130	107	102	54
Proportion ^a					0.78	0.49

^a $P < 0.0001$. Na-P: Sodium-phosphate; Prep.: Preparation; PEG: Polyethylene glycol. ¹Randomized controlled trial.

effects model. Forest plots were constructed for visual presentation of the individual studies proportions and the pooled proportion^[21].

RESULTS

Our search yielded 550 studies of capsule endoscopy, of which 8 investigated the preparation for small bowel capsule endoscopy and otherwise met the inclusion criteria^[10-17].

Five out of these studies compared the proportion of “good” scores between the groups^[10-13,17]. The findings are shown in Table 1. A total of 237 patients were included, 130 with and 107 without preparation.

Seven out of these studies included a comparison of gastric and small bowel transit times and in proportion to examinations of cecum demonstration^[10,11,13-17]. The findings are shown in Table 2. A total of 401 patients were included, 221 with and 180 without preparation.

A “good” score was similarly defined in the five papers found suitable for comparison of preparations. In our two papers^[10,11] we used a score of 3 grades, where “good” was defined as a good mucosal visualization in more than 80% of the small bowel transit time. Albert and coworkers^[12] used a score of 4 grades, and we used their scores 0 and 1 as “good”, when there was no limitation for interpretation. Viazis and coworkers used the term adequate when more than 90% of the small bowel transit time and the mucosa was clear^[17], and Ben-Soussan and colleagues, using a score of 4 grades, defined “good or excellent” when visibility was good in more than 75% of the small bowel transit time^[13]. A “good” score was documented for 78% of the patients with preparation (95% CI, 65%-88%) compared to 49% of the patients without preparation (95% CI, 35%-62%; $P < 0.0001$; Figure 1).

The capsule reached and visualized the cecum in 76% of the patients with preparation and 68% without. This difference did not reach statistical significance (Figure 2).

There was no statistically significant difference between the endoscopies performed with or without preparation in gastric transit time (pooled effect size, -0.054; 95% CI, -0.418 to 0.308) or small bowel transit time (pooled effect size, -0.327; 95% CI, -1.419 to -0.765; Figure 3A and B).

Table 2 List of 7 case-control studies, comparing transit times and proportions of cecum demonstration, fulfilling the inclusion/exclusion criteria ($n = 401$)

Author	Ref.	Prep.	No. with	No. without	GTT with (min)	GTT without (min)	SBTT with (min)	SBTT without (min)	Cecum reached with	Cecum reached without
Niv Y, 2004	10	Na-P	22	10	25.0	18.0	300	333	16	5
Viazis N, 2004 ²	17	PEG 2L	40	40	36.2	44.1	291.8	304.6	32	26
Niv Y, 2005	11	Na-P	23	23	25.0	40.0	341	241	18	18
Ben-Soussan E, 2005	13	PEG 2L	26	16	45.7	25.5	288	271	24	16
Fireman Z, 2005	14	PEG 1L or Na-P or Erythro	55 ¹	40 ¹	39.7	45.5	228	218	NM	NM
Dai N, 2005	16	PEG 4L	33	28	13.0	14.0	213	253	32	22
Caddy GR, 2006	15	Erythro	22	23	50.5	38.4	304.4	302.6	7	5
Total			166	140					129	92
Average \pm SD					33.5 \pm 13.2	32.2 \pm 12.8	280.8 \pm 44.9	274.7 \pm 40.6		
Proportion									0.76	0.68
<i>P</i>					0.296		0.155		0.38	

With: With preparation; Without: Without preparation; GTT: Gastric transit time; SBTT: Small bowel transit time; Na-P: Sodium-phosphate; Prep.: Preparation; PEG: Polyethylene glycol; NM: Not mentioned. ¹Not included; ²Randomized controlled trial.

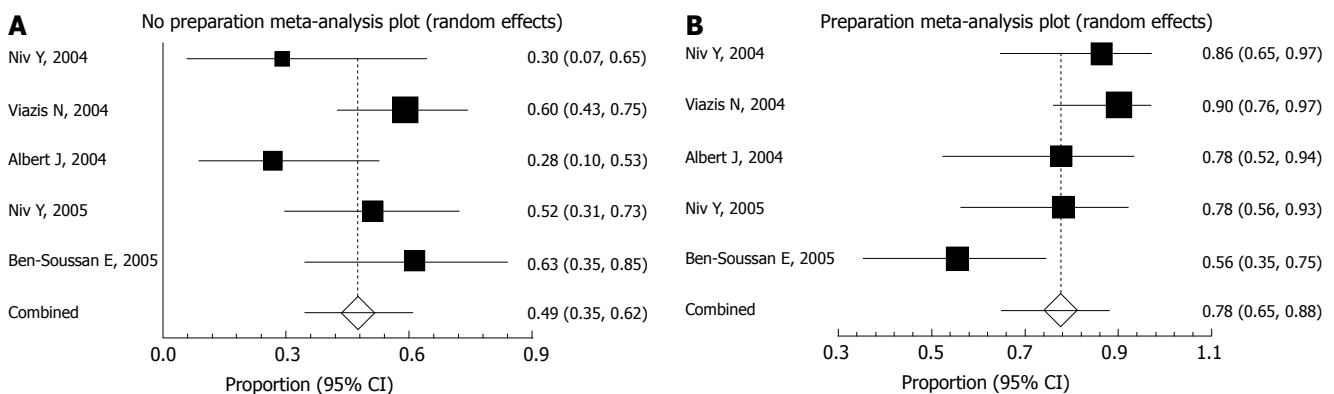


Figure 1 Proportion of a “good scoring” in small bowel visualization with (B) and without (A) preparation, a meta-analysis of 5 papers and 237 patients. Data were sufficiently homogenous, and statistically significant difference that favored preparation for good visualization is demonstrated. The statistical significance of results did not differ between fixed and random mode.

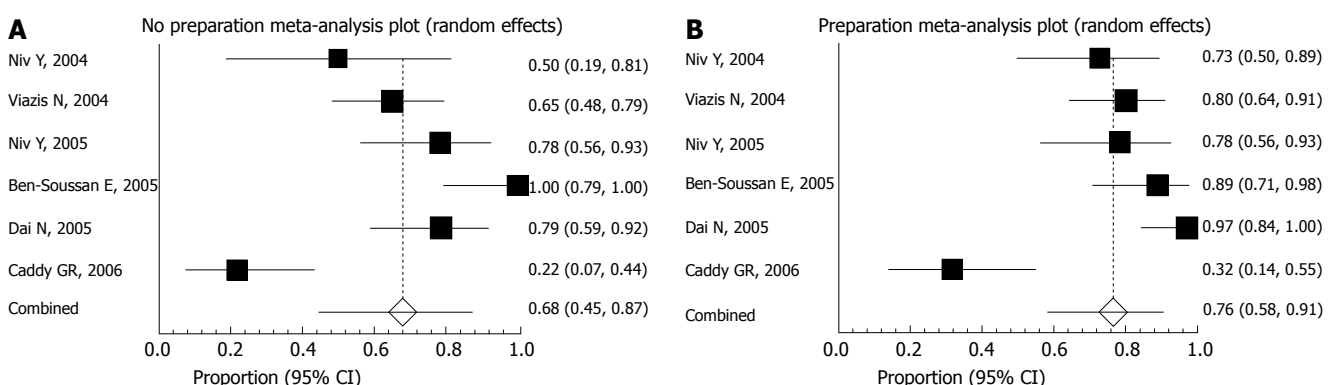


Figure 2 Proportion of cecum demonstration with (B) and without (A) preparation, a meta-analysis of 7 papers and 401 patients.

CONCLUSION

Capsule endoscopy is a new technology, and there is still no consensus regarding the proper preparation. The manufacturer recommends only a 12-h fast after 24-h intake of clear liquids. It may be in the interest of the manufacturer to recommend such a simple preparation, so that the procedure will be easy to perform. However,

since the procedure is costly, time consuming, and not usually repeated, it is critical to optimize the quality of visualization, which may be impaired by secretions, bubbles or coating of the capsule with intestinal residue.

Few studies have compared different preparation strategies, using different approaches, designs, and methods. Two controlled studies by our group found that the sodium phosphate method provided better

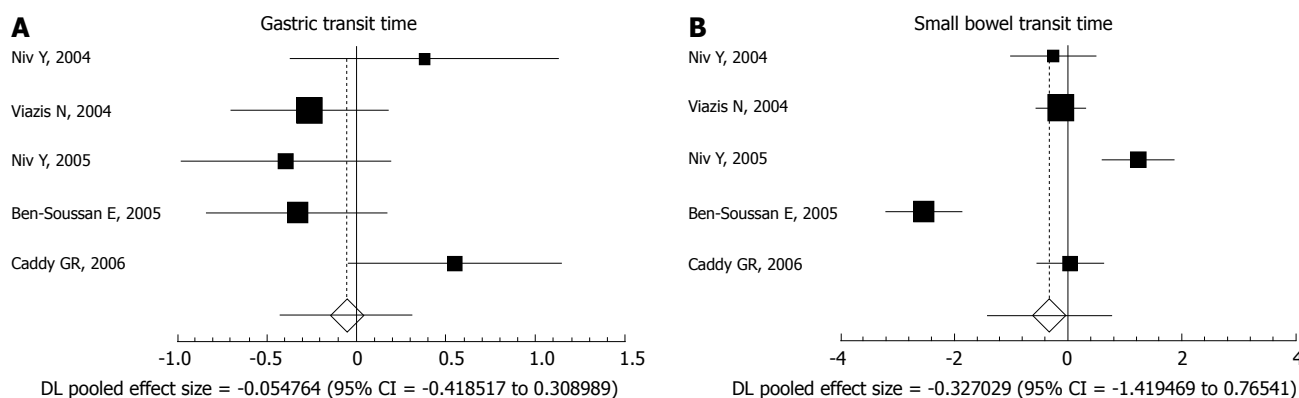


Figure 3 Differences in transit times in patients underwent preparation before the capsule examination and those who underwent the procedure without prior preparation, a meta-analysis of 7 papers and 401 patients. **A** = difference in gastric transit time, **B** = difference in small bowel transit time.

visualization than the standard method, with significantly less and later-appearing turbid fluid in the small bowel lumen, which could block the visual field of the capsule^[10,11]. Our results were in agreement with the prospective, randomized, controlled trial of Viazis *et al*^[17], which demonstrated a significant advantage of preparation with polyethylene glycol over fasting alone. The findings in our control group were also similar to the control findings of Viazis *et al*^[17] in terms of transit times, percentage of procedures in which the cecum was reached, and diagnostic yield (secondary endpoint). Taken together, the data from our nonrandomized comparison case-control studies^[10,11] and the randomized study of Viazis *et al*^[17] indicate that bowel preparation with either sodium phosphate or polyethylene glycol is superior to simple fasting for capsule endoscopy. Albert and colleagues used simethicone for bubble absorption and improved the visibility of the small bowel mucosa^[12]. Thus their paper was added to this meta-analysis even though simethicone is not a classic preparation drug and may not be considered equivalent to polyethylene glycol or to sodium phosphate^[12].

Only 3 papers looked at the diagnostic yields as a secondary endpoint, since it would be much more useful to compare more definite outcomes such as number and type of lesions visualized in attempting to compare the effect of preparation and no preparation^[11,13,17]. Ben-Soussan and colleagues found no association between preparation and diagnostic yield of the capsule examination^[13], while we and Viazis found a significant advantage of preparation for findings more lesions^[11,17]. Thus, this point is still controversial.

The main weakness of the study is in the different preparation regimens, and different scores used by the individual centers, sometimes addressed to evaluate different aspects of the small bowel cleansing: amount of intraluminal fluid, gas or air bubbles, and percentage of “free” mucosal surface.

Our search yielded 8 studies comparing preparation with sodium phosphate, polyethylene glycol or simethicone in one group of patients with simple fasting in another group, before capsule endoscopy of the small bowel. The results of the meta-analysis clearly demonstrated that although preparation improves the quality of small bowel visualization, it has no significant effect on gastric transit

time, small bowel transit time, or cecum demonstration. The main limitation of our meta-analysis is the diversity of the methods used by the different trials. All the studies were controlled, but only one of them was randomized^[17]. Two studies compared different strategies used by different centers, and 5 studies compared 2 different periods with changed strategies. Nonetheless, the study groups in every study were very similar in their demographic and clinical data. Most of the patients were referred for the capsule examination to evaluate occult gastrointestinal bleeding. Co-morbidity was also similar between the groups, and no significant alterations in transit times were anticipated. Thus, further studies are needed to definitively establish the association among motility factors, battery life-time, good visualization of the bowel, and completeness of the examination.

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Proton pump inhibitors and an emerging epidemic of gastric fundic gland polyposis

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Abstract

Fundic gland polyps are now commonly recognized during endoscopy. These polyps are benign, often multiple and usually detected in the gastric body and fundus. In the past, these polyps were sometimes associated with familial adenomatous polyposis. In recent years, it has become evident that increasing numbers of these polyps are being detected during endoscopic studies, particularly in patients treated with proton pump inhibitors for prolonged periods. In some, dysplastic changes in these polyps have also been reported. Recent studies have suggested that there may be no increase in risk of colon cancer with long-term proton pump inhibitor therapy. While temporarily reassuring, ongoing vigilance, particularly in those genetically predisposed to colon cancer, is still warranted.

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Key words: Gastric polyps; Fundic gland polyposis; Gastric dysplasia; Gastric cancer; Colon polyps; Familial polyposis coli; Adenomatous polyposis coli gene mutation

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GASTRIC POLYPS

Gastric polyps and tumors have been classified into

mucosal, lymphoid, mesenchymal and stromal types^[1]. Both neoplastic (adenoma, carcinoma, carcinoids) and non-neoplastic mucosal types occur. Most neoplastic epithelial polyps are asymptomatic and, usually, occur in the gastric antrum. Occasionally, these polyps bleed or present with an iron deficiency anemia. Rarely, gastric adenomas, particularly if large and pedunculated, cause symptoms from intermittent gastric outlet obstruction due to duodenal prolapse. Carcinoma may also complicate such adenomas. But, even these complex malignant polyps have been amenable to endoscopic resection^[2].

FUNDIC GLAND POLYPOSIS

One of the most common types of gastric polyps, generally classified as non-neoplastic, is the sporadic fundic gland polyp. Usually, these are confined to the gastric body and fundus and rarely cause symptoms. Fundic gland polyps are typically detected during investigation for abdominal pain, dyspepsia or chronic reflux. Historically, it has been estimated that these polyps occur in up to 2% of all endoscopic studies. Although quite characteristic, they were only first described as a distinct pathological type in 1977^[3]. Previously, these were reported to occur most often in females and were thought to be derived from the parietal cell- and chief cell-bearing region of the gastric mucosa^[1]. Cystic dilation of pits deep in the mucous neck cells was observed with mucous cells, chief cells and parietal cells lining these mucosal cysts. Inflammatory changes in these polyps is usually minimal or absent. It has been reported that these polyps may resolve spontaneously^[4].

FAMILIAL ADENOMATOUS POLYPOSIS (FAP)

Gastric polyps also occur in the majority of patients with FAP, screened with endoscopy^[5-7]. FAP results from inherited germline mutations in the adenomatous polyposis coli (*APC*) gene, coupled with second somatic mutations. This leads to inactivation of both copies of the *APC* tumor suppressor gene^[8,9]. Importantly, the most common gastric lesion in FAP is the syndromic fundic gland polyp, histologically similar to sporadic fundic gland polyps. Gastric and duodenal adenomas also occur in FAP, but are much less common. Fundic gland polyps are most often not associated with FAP and have little or no potential for malignant transformation^[5,10]. However,

high-grade dysplasia and gastric adenocarcinoma have both been associated with fundic gland polyps in FAP^[7,11-15]. As a result, colonoscopic surveillance has been suggested to determine if FAP or an attenuated form of FAP is present when fundic gland polyps are first detected^[16]. Evidence strongly supporting this approach is not available. As fundic gland polyps are being increasingly recognized during endoscopic evaluations, added colonoscopic studies might be best reserved for those with concomitant gastric or duodenal adenomas.

MOLECULAR GENETIC MARKERS

Since both sporadic and syndromic FAP-associated fundic gland polyps have similar histological appearances, genetic markers have been explored to determine if there are additional similarities or differences. Distinct disruptions in the Wnt signaling pathway with activating beta-catenin mutations occur with sporadic polyps, while FAP-associated polyps showed second, somatic mutations of the *APC* gene^[17]. With chronic acid suppression therapy, multiple fundic gland polyps develop that are also histologically and genetically identical to single sporadic polyps^[18]. In these, beta-catenin mutations were detected in most polyps. In addition, distinct mutations in different polyps from the same individual indicated a multifocal origin for the polyps^[18]. Separate studies on fundic gland polyps in the absence of FAP have also failed to show *APC* gene deletions^[19]. Interestingly, a very low prevalence of *H pylori* infection was also noted with these polyps^[19]. In contrast, *APC* gene mutations were found in FAP with both syndromic fundic gland polyps and high grade dysplasia in gastric epithelium^[20]. While these studies have served to elucidate molecular changes in sporadic and syndromic (FAP) gastric fundic gland polyps, more information is needed to determine if these markers could be used cost-effectively to predict risk in fundic gland polyps for eventual development of gastric cancer.

PROTON PUMP INHIBITOR-ASSOCIATED GASTRIC POLYPS

Omeprazole was first introduced for clinical use as a proton pump inhibitor in 1988. Since then, worldwide sales figures for proton pump inhibitors have dramatically risen with estimated sales now totaling over \$10 billion and rising. Over 720 million prescriptions for proton pump inhibitors have been written worldwide, largely for long-term use, while large randomized clinical trials have confirmed the high efficacy and safety profile of long-term treatment^[21]. In addition, however, substantial physiological changes occur with chronic acid suppression therapy. Increased levels of circulating gastrin occur. This hormone stimulates increased cell proliferation. Chronic ECL cell stimulation also results as reflected by increased levels of chromogranin A, an endocrine cell product^[22]. In recent years, gastric fundic gland polyps have become increasingly detected in patients on long-term proton pump inhibitor therapy^[23-26]. These fundic gland polyps are often multiple in this setting and localized in the gastric body and fundus.

No definite sex predilection has been defined. They appear to have similar histologic and genetic features to those developing without proton pump inhibitor use. Recent studies have defined a relationship between the length of drug use, especially after 12 mo, and increased polyp risk^[26]. In addition, most patients with fundic gland polyps on proton pump inhibitors are *H pylori*-negative^[26], consistent with a previous report of fundic gland polyp regression following acquisition of *H pylori*^[27]. Of concern, multiple fundic gland polyps have also been noted in some children on long-term omeprazole therapy^[28,29]. Moreover, in a pediatric FAP population on proton pump inhibitors for more than 6 mo, dysplasia was reported in fundic gland polyps^[29]. These studies imply that with increasing use of long-term proton pump inhibitors, an epidemic of fundic gland polyposis will be defined. Studies are needed to determine if further follow-up of patients on long-term therapy with proton pump inhibitors and fundic gland polyps is warranted. In spite of an early record of safety with long-term use, there remain concerns regarding the potential risk of cancer with long-term exposure, not only in those with FAP, but also in those genetically predisposed to cancer. This concern is reflected in the recently published studies on colon cancer risk with long-term proton pump inhibitor exposure. In these studies, no increased risk was shown^[30,31]. While temporarily reassuring, ongoing vigilance will be required before the final chapter is written.

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Feasibility of herpes simplex virus type 1 mutants labeled with radionuclides for tumor treatment

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Abstract

For over one hundred years, viruses have been recognized as capable of killing tumor cells. At present, people are still researching and constructing more suitable oncolytic viruses for treating different malignant tumors. Although extensive studies have demonstrated that herpes simplex virus type 1 (HSV-1) is the most potential oncolytic virus, therapies based on herpes simplex virus type 1 vectors still arouse bio-safety and risk management issues. Researchers have therefore introduced the new idea of treating cancer with HSV-1 mutants labeled with radionuclides, combining radionuclide and oncolytic virus therapies. This overview briefly summarizes the status and mechanisms by which oncolytic viruses kill tumor cells, discusses the application of HSV-1 and HSV-1 derived vectors for tumor therapy, and demonstrates the feasibility and prospect of HSV-1 mutants labeled with radionuclides for treating tumors.

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Key words: Oncolytic virus; Herpes simplex virus type 1; Mutant; Radionuclide; Tumor therapy

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INTRODUCTION

Oncolytic virus^[1], a tumor-specific replicating virus, is

one of a number of natural or engineered viruses that can selectively replicate in and cleave tumor cells, but not kill normal somatic cells. There are six major mechanisms by which viruses can lyse tumor cells: (1) Viruses can replicate, package in and cleave tumor cells; (2) Similar to non structure protein (NS protein), some protein particles produced by virus replication can induce host cell apoptosis; (3) Both nonspecific and specific immune responses as well as immunoreactive sensitivity to cytokine (TNF- α , IFN- γ) associated with virus infection can accelerate tumor cell lysis; (4) Virus associated antigen can enhance MHC- I expression on tumor cells, and increase the ability of antigenic specific cytotoxic T cells to kill tumor cells; (5) Oncolytic virus can enhance the sensitivity of tumor cells to radiotherapy and chemotherapy; and (6) Oncolytic virus can carry and express cancer therapeutic genes.

At present, many engineered oncolytic viruses have been used in animal or human studies. Some of them have been or are ongoing into phase III clinical trials, especially adenovirus. ONYX-015^[2,3], a derivative of adenovirus with a deletion of the E1B-55KD gene, can kill p53-deficient tumor cells, but not cells with functional p53. Many limitations were found, however, in its phase II clinical trial; ONYX-15 could neither treat tumors sufficiently, infect secondary tumor metastases efficiently, nor spread sufficiently in solid tumors^[2,3]. Nevertheless, evident organ impairment, toxicity and environmental events have not been found.

Several other oncolytic viruses (Newcastle disease virus, reovirus, retrovirus, rabies virus) have not been extensively used in research and clinical therapy, due to small genome size, limited host range, unclear replication mechanisms, etc.

RESEARCH AND CLINICAL APPLICATIONS OF HERPES SIMPLEX VIRUS TYPE 1 FOR TUMOR TREATMENT

Compared with other viruses, herpes simplex virus type 1 (HSV-1) has a number of favorable properties for cancer treatment^[4]. (1) In spite of its neurotropism, HSV-1 possesses a wide range of host cells, which is superior to adenovirus. (2) HSV-1 is able to infect dividing cells as well as nondividing cells. (3) Its DNA has been completely sequenced^[5], and the genome of 152 kb has exceeded adenovirus genome of 36 kb. Furthermore, transgenes can replace as much as 30 kb of the deleted HSV genome in replication-defective HSV-1 mutants, and

simultaneous delivery of multiple transgene and insertion of heterogenous promoters are allowed. (4) Its genome will not integrate into the cellular genome, resulting in little insertional mutagenesis, which is the major concern in retrovirus and adeno-associated virus vectors. (5) The entire replication cycle is usually about 20 h in permissive cells^[5], and that of adenovirus is 48-72 h^[6]. (6) Recombinant HSV-1 is easily engineered, and purified viruses with a high titer can be routinely prepared. (7) HSV-1 can efficiently infect different cells^[7], which is advantageous for the generalization from preclinical results to clinical trials. In contrast, the application of adenovirus vectors has been limited by few animal models. (8) HSV-1 infection is able to spread through both cell junctions and extracellular spaces and can penetrate into solid tumors with less systemic spread. (9) HSV-1 rarely causes life-threatening illness even in immune-competent adults. (10) HSV-1 infection can be treated using several anti-HSV-1 drugs (such as Acyclovir and Famciclovir)^[8].

Two main types of HSV-1 vectors are used in tumor therapy: replication-defective vectors and conditionally replication vectors. They can either terminate viral lytic infection or only confine themselves in certain types of cells.

Replication-defective vectors, in which either one of a few essential viral genes are deleted and then transgene expression cassettes are inserted in a viral genome, can effectively express transgene products, but are unable to replicate in cells unless host cells can supply the deleted viral functions. The HSV-1 mutant is used as a transgene in nerve cells. The HSV-1 thymidine kinase (TK) gene, a suicide gene encoded by UL23, is unique and most frequently used. TK protein can transform innocuous prodrugs such as GCV into cytotoxic drugs, resulting in termination of DNA synthesis in active cells, especially in tumor cells. Furthermore, bystander effects generated by TK/GCV systems are also toxic to tumor cells, but not towards neurons and quiescent glia^[9]. Besides the TK gene^[10], other cancer therapeutic genes such as connexin-43^[11], p53^[12], IL-2^[13], granulocyte-macrophage colony-stimulating factor (GM-CSF)^[13], IL-12^[14] and interferon (IFN- γ)^[15], have also been delivered in the replication-defective HSV-1 vectors for treating malignant gliomas and other types of tumors, and an antitumor effect has been demonstrated by intratumoral injection in animal models. In addition, multiple therapeutic genes, for example, co-expressing TK and TNF- α ^[16], TK, connexin-43 and TNF- α ^[17], TK and IL-12^[18], have simultaneously been delivered in replication-defective HSV-1 vectors in extensive studies. As a result, tumor growth was significantly inhibited.

Conditionally replicating vectors, generated by deleting some nonessential viral genes, can preferentially infect, replicate in, and lyse tumor cells. Therapeutic transgenes can also augment the antitumor effect.

Recently, there have been many reports about HSV-1 mutants. In several animal tumor models, HSV-1 mutants with TK gene deletion were created and tested for oncolytic virotherapy, which could induce tumor regression following intra-neoplastic administration^[19]. The UL39-

deleted virus, hrR3, efficiently replicates in malignant cells but proliferates less in normal cells^[20]. It was demonstrated that hrR3 had produced significant antitumor effects and survival benefits in animal models such for brain, colon, pancreas and liver cancers. In animal studies, HSV1716^[21], R3616^[22,23] and R4009^[22,23], which are the ICP34.5-deleted strains, are able to replicate when inoculated into neuron and other non-replicating cells. In contrast, in animal models of glioma, mesothelioma, melanoma, ovarian and lung cancer, HSV1716, R3616 and R4009 are replicated significantly. G207^[24,25], containing deletions of both copies of ICP34.5 gene and a insertion of *E. coli* lacZ gene in UL39 gene which encodes ICP6, had some favorable properties for cancer treatment. Competent replication in tumor cells, attenuated neurovirulence, GCV hypersensitivity, temperature sensitivity and the product of lacZ gene were easily detectable. G47 Δ ^[26], a derivative of G207 with an additional ICP47 gene deletion, could more efficaciously inhibit tumor growth *in vivo* than its parent G207, while safety was unaffected^[26]. Under the name of NV1020^[27], R7020^[27,28] contained a 15kb deletion of ICP34.5, the deletions of UL24, UL55, UL56 and endogenous TK gene and the insertion of exogenous TK gene which was controlled by α 4 promotor. Compared with R3616^[22,23] (double ICP34.5-deletion mutant), R7020 was more efficacious in inhibiting the growth of tumors, and more sensitive to ACV and GCV. Myb34.5^[29] had the deletion of both endogenous copies of the ICP34.5 gene and re-insertion of this gene into the ICP6 locus, with the new ICP34.5 gene under the control of the B-myc promoter. In contrast to hrR3, Myb34.5 was active in tumor cells, but was more attenuated in normal cells.

Although the usefulness of HSV-1 vectors for treating tumors have been confirmed, there are some limitations in their application. (1) It is more difficult to produce multiple gene deleted HSV-1 vectors than the wild-type virus, resulting in a lower yield. The opportunity of homologous recombination of wild-type HSV-1 with recombinant viral mutants is concerned. (2) It is particularly difficult to keep long-term stability (over 6-mo period) of HSV-1 derived vectors, either in aqueous solution or in lyophilized form. (3) The virus may rapidly spread in individuals with immunodeficiency. The efficacy may be reduced by immune response induced by antiviral or antitransgene products. (4) Attention must be paid to the safety in use of HSV-1 because HSV-1, a human pathogen, has broad cell type tropism and high replication capacity. So the safety and efficacy of HSV-1 mutants for tumor treatment are still a focus of future studies.

SUPERIORITY AND PROSPECT OF HSV-1 MUTANTS LABELED WITH RADIONUCLIDES FOR TUMOR TREATMENT

HSV-1 mutants can selectively infect tumor cells, but not suppress the growth of normal cells. There may be a synergistic antitumor effect, when they are labeled with radionuclide. With the help of the viral vectors, radionuclide will be carried into tumor tissues and bring

about radiation damage to tumor cells. It is rarely reported whether HSV-1 can be labeled with radionuclide, whether viral bioactivity may be influenced by the virus labeled or radionuclide which will enter into tumor cells together with virus. We analyzed these issues in this overview.

HSV-1 has an enveloped double-stranded nucleic acid (dsDNA). Located in the core of the virus, dsDNA is surrounded by a protein shell called a capsid which consists of 162 capsomeres arranged in a $T = 16$ icosahedral symmetry. The channels which are controlled by tegument proteins are contained in the capsid, controlling the transport of dsDNA through the channel. Surrounding the capsid, an amorphous tegument contains at least eight types of proteins that play an important role during HSV-1 infection. An outer envelope is composed of lipid bilayer with about 13 different viral glycoproteins.

It is well known that the HSV-1 envelope and tegument-capsid surrounding dsDNA consist of multiple proteins, about 60%-80% protein of the whole structure elements. However, HSV-1 mutants are generally constructed by modifying the dsDNA without altering their tegument-capsid. There are enough proteins, which well fit the labeling. Furthermore, the proteins such as monoclonal antibody, polypeptide and ligand, can all be labeled successfully^[30,31]. As for radionuclide, there are ^{125}I , ^{131}I , ^{32}P , ^{35}S , $^{99\text{m}}\text{Tc}$, ^{188}Re , ^{186}Re , ^{90}Y and so on. In a word, the proteins contained in HSV-1 structural elements are considerably suitable for radiolabelling.

During initial infection, HSV-1 attaches first to the host cell surface receptor, and fuses with the membrane of host cells, and the de-enveloped tegument-capsid is efficiently and rapidly transported to the nuclear pore complexes, where the viral dsDNA is released. The capsid with associated tegument structures can also be carried to the nuclear pore through the microtubules^[32]. The transition from virtual attachment to penetration is very rapid and takes only several minutes^[33]. At the early phase of virus infection, the viral envelope glycoproteins, gC and gB, bind to cell surface heparin sulfate^[34]. Then, viral glycoprotein gD binds to certain cell surface receptor (e.g., nectin-1a, nectin-1b, 2a, 2d, HveA), contributing to virion-cell fusion^[35]. Besides, three other viral envelope glycoproteins, gB, gH and gL, have been shown to be helpful for the HSV-1 penetration into host cells^[36]. As a result, the viral proteins are delivered into different metastructures. So, it is feasible that HSV-1 vector mutants could carry radionuclide-labeled proteins into tumor cells.

Could radiation from radiolabeling of viruses destroy their structures and damage their biologic characteristics? A study^[37] on the combination of gene therapy with radiotherapy for tumors revealed that the gene products in mice lung cancer cells had increased dose-dependently, when irradiated by gamma-ray at doses of 2-40 Gy followed by AdCMVlu transfection. The efficacy could be up to 24 times higher, and tumor growth was inhibited. Kanazawa^[38] demonstrated that the number of replicative dsDNA of the laryngeal cancer cell line (HEp-2) and Henrietta Lacks (HeLa) strain of cancer cells had a significant enhancement in the combination of virus system and radiotherapy. The virus system was composed of adeno-associated virus (AAV) vectors,

the HSV-1 thymidine kinase (HSV-tk) and ganciclovir (GCV). The radiation dosage was 4Gy. These experiments *in vitro* suggested that the combination of AAVtk/GCV system with radiotherapy was significantly effective in the treatment of cancers. Weichselbaum RR^[39] found radiation could induce the transcription of CarG elements in the Egr-1 promoter sequences which lies the upstream of TNF- α cDNA. At the same time, TNF- α obtained a high expression. Generally, radiotherapy would stimulate the virus replication, resulting in a higher HSV-1 antitumor activity^[40]. Other studies had also indicated that ionizing radiation could enhance anti-cancer activity of hrR3 without altering its replication^[40]. Similarly, a synergistic antitumor response, with more tumor regression and better survival rate, was induced by the combination of ionizing radiation and R3616 deleted ICP34.5^[41]. So, radionuclide labeled to HSV-1 mutants does not damage the viral biological function, but can enhance the antitumor effect.

It is extremely important that compounds labeled with radionuclide should possess a good stability *in vivo*. The labeled compounds should stay in the tumor cell cytoplasm while the HSV-1 mutant dsDNA enters the nucleus. HSV-1 recombinant-labeled radionuclide should, therefore, have a favourable stability before penetration into the tumor cell nucleus. Of course, suitable radiolabeling methods which can keep labeled compounds stable should be well considered. Diethylenetriaminepentaacetic acid (DTPA) is known as one of the difunctional chelators. This kind of chelator has a stereochemical structure group which is found to tightly hold radionuclide and amino acids fragments active group by a covalent link. Thus, radionuclide (^{188}Re , ^{90}Y) is indirectly linked up with protein molecule, and the compounds labeled with radionuclide is stable *in vivo*. Moreover, the biochemical activity of radiolabeled complex will not be influenced. As a chelating agent, DTPA has a potential and broad applicability^[30,31].

As far as radiolabeling and the structure of HSV-1 are concerned, the feasibility of labeling radionuclide to HSV-1 mutants is demonstrated by a plenty of animal experiments and clinical trials. Furthermore, once the radionuclide is introduced, nuclear medicine approaches may be employed in the clinic to monitor and assess the therapeutic efficacy *in vitro* in a non-invasive manner.

In order to increase the efficiency of HSV-1 mutants in killing tumor cells, we intend to construct HSV-1 GRT, by deleting ICP 47 gene and one copy of ICP 34.5 gene and inserting *E. coli* Z gene and TNF α gene in UL39 gene. Then HSV-1 GRT can be labelled with ^{131}I or ^{188}Re directly or indirectly. We will study the expression of HSV-1 GRT or radioactive compounds (^{131}I -HSV-1 GRT, ^{188}Re -HSV-1 GRT and ^{188}Re -DTPA-HSV-1 GRT) in tumor cells and mechanism of inhibiting or killing tumor cells. In other words, our aim is to develop an ideal drug which can combine viral oncolysis with radionuclids therapy. With the help of the HSV-1 GRT, radionuclids (^{131}I or ^{188}Re) can be carried into tumor tissues and bring about radiation damage to tumor cells.

CONCLUSION

HSV-1 mutants provide a choice for oncolytic viruses

to selectively target and kill tumor cells, and they are also attractive vectors helping radionuclide get to focal tumor tissues. The combination of viral oncolysis with radionuclide therapy will achieve a synergistic anticancer effect, with more inhibition of tumor growth, less toxicity and fewer side effects than either HSV-1 mutants or radionuclide therapy. It is reasonably concluded that HSV-1 mutants labeled with radionuclide will be a potential focus for tumor treatment.

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TOPIC HIGHLIGHT

Alan BR Thomson, MD, Series Editor

Management of inflammatory bowel disease in the pregnant patient

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Abstract

Inflammatory bowel disease (IBD) is a chronic disorder affecting young adults in their reproductive years. Many young women with IBD express concern about the effect their disease will have on fertility, pregnancy course and fetal development. This article presents an approach to management of IBD in the pregnant patient, including counseling and investigation, and summarizes existing data on the safety of medications used to treat IBD in pregnancy and breastfeeding.

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Key words: Inflammatory bowel disease; Ulcerative colitis; Crohn's disease; Fertility; Pregnancy; Breastfeeding

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INTRODUCTION

Inflammatory bowel disease (IBD) is a chronic disorder affecting young adults in their reproductive years. Voluntary childlessness in patients with IBD is greater than in the

general population due to relationship difficulties, body image problems, fear of pregnancy and inappropriate medical advice that pregnancy might be dangerous^[1-3]. Pregnancy planning should be discussed with the patient and her partner early on prior to conception. Education and open communication are important as patients may be reluctant to broach this topic on their own. Patient concerns often relate to fertility, impact of IBD on pregnancy and fetal development, and drug effects pre-conception, during pregnancy and while breastfeeding.

FERTILITY

Women with ulcerative colitis (UC) who have not undergone surgery or those with inactive Crohn's Disease (CD) have fertility rates comparable with the rest of the population. In comparison, women with UC who have had surgery^[4] or those with active CD^[5,6] have increased infertility. Fertility appears to revert to normal after induction of remission in women with CD. Women who have their first pregnancy after the onset of IBD have fewer pregnancies than population controls, whereas women who became pregnant prior to onset of IBD have similar reproductive history^[5]. In addition, women with CD have a delayed age of first pregnancy after being diagnosed^[7] and have been shown to have fewer children than might be expected after diagnosis with a higher rate of failure to conceive^[8].

IMPACT OF IBD ON PREGNANCY AND FETAL DEVELOPMENT

The initial impression looking at multiple, small observational studies of pregnancy in women with IBD was that the outcome was normal^[6,9]. Fetal mortality risk (spontaneous abortion, stillbirth or neonatal death) is not higher for IBD patients^[3], but there is an increased risk of preterm delivery (< 37 wk) and low birth weight (< 2.5 kg) in mothers with IBD^[10-14]. Still, the majority of women with IBD will have a normal outcome of pregnancy. Disease activity is the main adverse factor predisposing to prematurity and low birth weight babies^[15].

DISEASE ACTIVITY

Approximately one-third of women with inactive IBD at conception will relapse during the pregnancy or puerperium.

This risk of a flare is no greater than any other year of the patient's life^[13]. If conception occurs at a time when IBD is active, disease will settle only in about one-third of women with UC or CD^[12,16]. One-quarter of patients with active IBD during pregnancy will experience chronically active disease and in about half of these patients, disease will worsen (45% UC, 33% CD)^[15]. Active disease has been associated with miscarriage, stillbirth, prematurity and low birth weight^[17]. Thus, conception is advised when IBD is in remission.

Maintenance therapy, if possible, should be continued throughout pregnancy and flare-ups of disease should be investigated and treated appropriately as in the non-pregnant patient. If conception occurs with active IBD, inducing remission with medical therapy carries less risk than continuing pregnancy without treatment.

TNF- α AND PREGNANCY LOSS

Tumour necrosis factor is a pivotal pro-inflammatory cytokine in IBD^[18]. Active IBD, more so CD than UC, is associated with elevated TNF- α levels^[19,20]. In the past, TNF- α has been thought to be primarily involved in triggering immunological pregnancy loss. Newer evidence suggests that the possible essential function of TNF- α is to boost death signaling to kill the embryo if damages triggered by detrimental stimuli will result in birth of offspring with structural anomalies^[21]. This theory seems more plausible from a clinical perspective since risk of pregnancy loss is higher with active IBD.

COUNSELLING THE IBD PATIENT WITH POTENTIAL FOR PREGNANCY

Preconception counseling

As with all patient-physician interactions, the therapeutic alliance, education and communication are important components in assisting the patient with IBD to make decisions about conception and pregnancy. Early on in the patient-physician relationship, preferably prior to conception, the physician should address fertility, IBD impact on pregnancy outcomes, medication effects and importance of disease remission in minimizing fetal risk. Many women want to be drug free during pregnancy and should not feel guilty about making this choice as long as it is an informed decision. The patient, her partner and her physician should discuss the possibility of disease exacerbation during pregnancy while off treatment and the necessary courses of action in such an event. Treatment choices depend on individual preference, disease severity and potential for drug toxicity. Risks and benefits of maintenance therapies during pregnancy with the best available evidence should be addressed. In addition, breastfeeding should be presented as a favorable option since it confers numerous benefits to both mother and child. The likelihood of medication secretion in breast milk and impact on fetal wellbeing become essential topics.

Alcohol

Patients contemplating pregnancy should be educated about the adverse effects of alcohol on fetal development.

Maternal consumption of alcohol during pregnancy can result in fetal alcohol syndrome (FAS), a permanent birth defect clinically defined by growth deficiency, central nervous system damage and dysfunction, and a unique cluster of facial abnormalities. Although FAS is the most extreme and recognizable expression of the adverse effects of alcohol on the fetus, prenatal alcohol exposure can also cause less pronounced mental, learning and behavioral disabilities in the child, commonly termed as Fetal Alcohol Spectrum Disorders (FASDs)^[22].

Smoking

Tobacco smoking during pregnancy has been associated with placenta previa, placental abruption, premature rupture of membranes, preterm birth, intrauterine growth restriction and sudden infant death syndrome (SIDS)^[22]. Smoking cessation should be encouraged in the patient prior to, during and after pregnancy.

Dietary supplementation and nutritional therapy

Folic acid, calcium and vitamin D: Folic acid supplementation is recommended for all pregnant women. Women with IBD may have folic acid deficiency or be taking medications that interfere with folic acid metabolism such as sulfasalazine^[15]. Thus, pregnant women with IBD should be encouraged to take 5 mg of folic acid per day instead of 1 mg/d as recommended for the general population. Also, patients with IBD on steroid therapy should be encouraged to take calcium and vitamin D supplementation to prevent bone loss.

Nutritional therapy: The average weight gain during pregnancy is 11-16 kg. Early nutritional intervention is indicated in pregnant women with active IBD who may not be gaining weight. Enteral feeding has been anecdotally shown to be associated with normal pregnancy outcome^[23] and total parenteral nutrition (TPN) may be required in very sick IBD patients.

Mode of delivery

In general, the decision to have a caesarean section should be made on purely obstetric grounds. Some surgeons advise elective caesarean section to avoid risk of anal sphincter damage^[15]. Vaginal delivery and episiotomy may lead to development or worsening of perianal CD^[24]. Current indications for caesarean section are active perianal disease and presence of an ileoanal pouch. There is no absolute contraindication to vaginal delivery in pregnant patients with inactive IBD.

RADIOLOGIC AND ENDOSCOPIC INVESTIGATION OF IBD EXACERBATIONS DURING PREGNANCY

Radiology

Radiographic imaging should be avoided unless obstruction, perforation or toxic megacolon are suspected. Investigations that expose the patient to less radiation are preferable - specifically plain abdominal films rather than CT or barium studies. Ultrasound is the safest form

Table 1 Food and drug administration (FDA) classes in pregnancy

Class	Definition
A	Controlled studies in women fail to demonstrate a risk to the fetus in the first trimester (and there is no evidence of risk in later trimesters) and the possibility of fetal harm appears remote
B	Either animal reproduction studies have not demonstrated a fetal risk, but there are no controlled studies in pregnant women OR animal reproduction studies have shown an adverse effect (other than decrease in fertility) that was not confirmed in controlled studies in women in the first trimester (and there is no evidence of risk in later trimesters)
C	Either studies in animals have repeated adverse effects on the fetus (teratogenic, embryonic or other) and there are no controlled studies in women or studies in women and animals are not available. Drugs should be given only if the potential benefit justifies the potential risk to the fetus
D	There is positive evidence of human fetal risk but the benefits from use in pregnant women may be acceptable despite the risk (e.g. if the drug is needed in a life threatening situation or for a serious disease for which safer drugs cannot be used or are ineffective)
X	Studies in animals or human beings have demonstrated fetal abnormalities OR there is evidence of fetal risk based on human experience OR both, and the risk of the use of the drug in pregnant women clearly outweighs any possible benefit. The drug is contraindicated in women who are or may become pregnant

of radiologic imaging - it can be used to assess abscess formation and can provide information on bowel wall thickness. MRI studies are also safe and have been used to diagnose terminal ileal CD during pregnancy^[17]. Since active disease in the mother has an adverse effect on the fetus, investigation for diagnostic and therapeutic purposes is warranted and should not be delayed.

Endoscopy

Endoscopy does not increase risk of premature labor or fetal abnormalities. Colonoscopy and sigmoidoscopy are safe during pregnancy if indicated^[25] and rigid sigmoidoscopy and rectal biopsy can be performed without fetal risk^[16]. Fetal heart rate should be monitored closely during the procedure and if required, sedation for endoscopy is thought to be safe. Antispasmodics such as hyoscine butylbromide (Buscopan) are contraindicated^[15].

MEDICAL/DRUG THERAPY

The most common medication classes used in treatment of IBD are presented below, along with US Food and Drug Administration (FDA) classifications for their use. Please refer to Table 1 for details of the FDA Drug Classification System and to Tables 2 and 3 for summaries of drug safety during pregnancy and breastfeeding respectively.

5-aminosalicylic acid preparations (FDA Class B)

This class of medications includes sulfasalazine, mesalamine, olsalazine and balsalazide. Aminosaliclates are used for maintenance therapy or induction of remission in IBD. All aminosaliclates are FDA category B except olsalazine which is FDA category C. Both sulfasalazine and mesalamine are safe in breastfeeding mothers.

Table 2 FDA classes of medications used to treat IBD

FDA class	Medications
B	5-Aminosalicylic acid preparations (sulfasalazine, mesalamine, balsalazide); metronidazole, amoxicillin/clavulanic acid; infliximab; adalimumab
C	5-Aminosalicylic acid preparations (Olsalazine); fluoroquinolones; corticosteroids; bisphosphonates; cyclosporin; tacrolimus
D	Azathioprine and 6-MP
X	Methotrexate; thalidomide

Table 3 Breastfeeding safety of medications used to treat IBD

Safe	Limited data, potential toxicity	Contraindicated
5-ASA preparations (sulfasalazine, mesalamine)	Metronidazole Fluoroquinolones	Thalidomide
Amoxicillin/clavulanic acid	Bisphosphonates	Methotrexate
Corticosteroids	Azathioprine	Cyclosporine (CsA)
	6-mercaptopurine (6-MP)	Tacrolimus (FK506)
	Adalimumab	
	Infliximab	

Sulfasalazine: Sulfasalazine competitively inhibits the brush border enzyme folate conjugase^[17]. It is a folic acid antagonist that theoretically can cause neural tube defects, cardiovascular and urinary tract abnormalities, and oral clefts^[26]. Clinical studies show no increase in incidence of congenital anomalies with sulfasalazine treatment^[27]. Given the effect of sulfasalazine on folic acid metabolism, extra folic acid supplementation is recommended for all pregnant women on this medication during the course of their pregnancy. Sulphasalazine and its metabolite sulphapyridine both cross the placenta and are secreted in breast milk at levels approaching that of maternal levels^[15]. Initial concerns about sulphasalazine in the neonatal setting were raised because a related sulfonamide (sulphisoxazole) could cause kernicterus in neonates^[28]. Several studies have shown that there is no increased incidence of neonatal jaundice with sulfasalazine^[9,30]. It is safe in breastfeeding mothers unless the fetus suffers from pre-existing hemolysis or if a rhesus incompatibility between mother and fetus is suspected^[17].

5-ASA (Mesalamine): Mesalamine is safe in pregnancy in conventional doses. Higher doses of greater than 3 g/d carry a potential risk of fetal nephrotoxicity, specifically interstitial nephritis^[30]. There is no significant change in congenital abnormalities, abortions or fetal distress with conventional 5-aminosalicylic acid treatment^[31-33]. Mesalamine is excreted in breast milk, but does not pose a significant risk to the baby^[34].

Antibiotics

Metronidazole and the quinolones have limited benefit for long term treatment of IBD. Short courses of these medications in treatment of pouchitis and perianal disease are low risk in the pregnant patient.

Metronidazole (FDA Class B): Metronidazole is used for treatment of active CD as well as perianal disease. This medication does not increase risk of spontaneous abortion or congenital anomalies^[35,36], although infants of women exposed to metronidazole in the second to third months of pregnancy have shown higher rates of cleft lip with or without cleft palate^[37]. Potential toxicity exists for long-term metronidazole use while breastfeeding^[38].

Fluoroquinolones (e.g. Ciprofloxacin) (FDA Class C): Human studies with fluoroquinolones have not shown increase in spontaneous abortion or congenital abnormality incidence^[39]. However, animal studies demonstrate musculoskeletal abnormalities induced by this medication class^[40]. Fluoroquinolones have a high affinity for bone tissue and cartilage, and may cause arthropathies in children. Although they are thought to have minimal risk overall, they should be avoided in the first trimester. Fluoroquinolones are likely compatible with breastfeeding, although data is limited.

Amoxicillin/clavulanic acid (FDA Class B): Amoxicillin/clavulanic acid is used as an alternative antibiotic for pouchitis. It does not confer increased teratogenic risk and is compatible with breastfeeding^[41].

Corticosteroids (FDA Class C)

Corticosteroids come in parenteral, oral and topical formulations. They include prednisone, prednisolone, dexamethasone and budesonide, and are indicated in moderately to severely active disease^[3]. Although corticosteroids cross the placenta, these agents pose a very small risk to the developing infant when used in the first trimester. This risk is often outweighed by the benefit of controlling the mother's IBD. Animal studies show increased frequency of cleft lip and cleft palate^[42]. However, these findings do not correlate with human results. A prospective controlled study in 2004 did not demonstrate increased rate of congenital anomalies or oral cleft with corticosteroid treatment during pregnancy^[43]. In addition, studies of corticosteroid use in pregnant patients with rheumatoid arthritis, antiphospholipid syndrome and SLE show no convincing evidence of teratogenesis^[44-46] and steroid use in IBD patients is not associated with pregnancy complications^[29].

Although adrenal suppression among neonates born to mothers taking corticosteroids is a theoretical concern, it has not been an issue in practice. Rectal preparations can be used until the third trimester unless there are specific concerns regarding miscarriage or premature delivery^[15].

Corticosteroids are secreted into breast milk in low concentrations. The maternal: fetal ratio of steroid serum concentrations depends on which steroid the patient is taking. Fluorinated steroids, betamethasone and dexamethasone are less efficiently metabolized by the placenta compared to prednisolone, resulting in fetal levels 10%-12% of that in maternal serum^[47]. While breastfeeding is safe with steroid use, mothers are encouraged to defer breastfeeding until 4 h after taking oral dosing of steroids to reduce neonatal exposure^[48].

There is no data on the safety of oral budesonide

in pregnancy. Inhaled or intranasal budesonide is not associated with adverse fetal outcomes. Budesonide effects on breastfeeding are unknown^[38].

Bisphosphonates (FDA Class C)

IBD patients on long term corticosteroid treatment may be started on bisphosphonates to prevent bone loss or treat osteoporosis. Alendronate and risedronate are two common bisphosphonates used to this effect. Alendronate has been shown in animal studies to cross the placenta and incorporate into fetal bone^[49]. Long-term effects of alendronate on human bone development are unknown and the half-life of alendronate is greater than 10 years. The concern with long-term bisphosphonate treatment is that the drug is slowly released from maternal bone and may result in continuous low-level exposure to the fetus during gestation. Thus, bisphosphonates should be used with caution in young women who have the potential for pregnancy.

Immunomodulators

Methotrexate (FDA Class X): Methotrexate is used as an alternative to azathioprine in treatment of steroid dependent or resistant CD. It is a teratogen that interferes with folic acid metabolism and purine synthesis. In obstetrics, it is used therapeutically to abort ectopic pregnancies^[50]. Fetuses exposed to methotrexate develop congenital anomalies collectively known as methotrexate embryopathy or fetal aminopterin syndrome characterized by intrauterine growth retardation, decreased ossification of the calvarium, hypoplastic supraorbital ridges, small low-set ears, micrognathia, limb abnormalities and sometimes mental retardation. Methotrexate is contraindicated in pregnancy and breastfeeding. Female and male patients on methotrexate must be using a reliable form of contraception and should avoid conceiving for 6 mo after stopping the drug. A woman who conceives on methotrexate and refuses therapeutic abortion should stop the methotrexate and immediately start high dose folic acid replacement^[51].

Azathioprine/6-mercaptopurine (6-MP) (FDA Class D): Azathioprine (a prodrug of 6-MP) and 6-MP are used in treatment of steroid-dependent or resistant IBD. Most evidence for safety of azathioprine in pregnancy comes from studies in transplantation. The fetus is protected from potential teratogenic effects of azathioprine and 6-MP since the fetal liver lacks the enzyme inosinate phosphorylase which is necessary to convert azathioprine and 6-MP to active metabolites. Both these medications when used in small doses in clinical practice do not affect human interstitial cell function or gametogenesis^[52,53]. Renal transplant patients and SLE patients on treatment with azathioprine and 6-MP have had good outcomes^[15]. Also, retrospective studies have also shown that these medications are safe in pregnant patients with IBD^[54,55]. If a patient is established on azathioprine or 6-MP therapy and the drug is essential to maintain remission, the patient should continue treatment. Breastfeeding is not recommended by the manufacturers of azathioprine and 6-MP, but clinical experience of mothers shows no adverse side effects^[15].

Cyclosporine (CsA) (FDA Class C): CsA is used to delay surgery in severe UC. It is a selective immunosuppressant with the ability to inhibit activation of T cells, preventing formation of IL-2. Although it is not teratogenic, it crosses the placenta and is secreted in breast milk at high concentrations. CsA is contraindicated with breastfeeding to avoid neonatal immunosuppression. It is most likely safe with no adverse fetal effects during pregnancy. Most of the data regarding the use of cyclosporine comes from transplant patients^[48,56]. CsA is a highly toxic drug for the mother with risk of hypertension, nephrotoxicity and hepatotoxicity. CsA should not be used during pregnancy, except to prevent urgent colectomy in patients with fulminant UC^[57].

Tacrolimus (FK506) (FDA Class C): Tacrolimus is a fungus-derived immunosuppressant with a mechanism of action similar to CsA. It has been associated with increased incidence of perinatal hyperkalemia and prematurity^[58]. A single case report with favourable outcome for a pregnant patient with UC was published in 2005^[59]. It is contraindicated in breastfeeding due to high concentrations in breast milk.

Thalidomide (FDA Class X): Thalidomide is used in treatment of refractory CD. It has extensive teratogenic sequelae including limb defects, central nervous system effects and abnormalities of the respiratory, cardiovascular, gastrointestinal and genitourinary systems. Thalidomide is contraindicated during pregnancy and breastfeeding. Women of childbearing age taking thalidomide should use two methods of contraception 1 mo prior to starting therapy, during therapy and for 1 mo after stopping therapy.

Biological therapy

Infliximab (FDA Class B): Infliximab is a chimeric monoclonal antibody that inhibits TNF- α , a pro-inflammatory cytokine. It is used in treatment of patients with severe active CD. One study and a patient series have provided data for safety of infliximab in pregnancy. The Infliximab Safety Database maintained by Centocor (Malvern, Pennsylvania, USA) presented outcome data on 96 women with direct exposure to infliximab. The outcomes for these women were not different from those of the general population^[60]. A patient series of ten women on maintenance infliximab throughout pregnancy ended in live births with no congenital malformations for all women^[61]. Still, long-term implications of in-utero exposure to infliximab remain unknown. Infliximab crosses the placenta but is not detectable in breast milk and case reports of women who breastfed while on infliximab do not suggest toxicity^[62]. However, long-term effects on the developing infant's immune system while breastfeeding on infliximab are unknown.

Adalimumab (FDA Class B): Adalimumab has been recently demonstrated as a safe and efficacious therapy in induction of remission and maintenance therapy in CD^[63]. There is one case report of a successful pregnancy in a woman with longstanding CD who began adalimumab

1 mo prior to conception^[64]. There is no data on long-term effects of adalimumab on the developing fetus, or on its safety in breastfeeding.

Fish oil supplements (FDA Class-not applicable): Fish oil supplements are used by some patients with IBD as an adjunct to medical therapy. A randomized controlled trial of fish oil supplementation demonstrated prolongation of pregnancy without detrimental effects on growth of the fetus or course of labour^[65]. Fish oil supplements are not rated by the FDA since they are not classified as a drug.

CONCLUSION

Mothers with IBD have an increased risk of preterm delivery (< 37 wk) and low birth weight (< 2.5 kg) babies. Active IBD has also been associated with miscarriage and stillbirth. Maintenance therapy for IBD, if possible, should be continued throughout pregnancy and flare-ups of disease should be investigated and treated appropriately as in the non-pregnant patient. If conception occurs with active IBD, inducing remission with medical therapy carries less risk than continuing pregnancy without treatment. Early on, the physician should address fertility, IBD impact on pregnancy outcomes, medication effects and importance of disease remission in minimizing fetal risk with female patients of child-bearing age. It is important to provide patients with careful counseling regarding potential teratogenicity or adverse outcomes of medication use during pregnancy and breastfeeding. Individual patient preference, disease severity and potential for drug toxicity should determine the best therapy choice for each patient.

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Isolation and bioinformatics analysis of differentially methylated genomic fragments in human gastric cancer

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Abstract

AIM: To isolate and analyze the DNA sequences which are methylated differentially between gastric cancer and normal gastric mucosa.

METHODS: The differentially methylated DNA sequences between gastric cancer and normal gastric mucosa were isolated by methylation-sensitive representational difference analysis (MS-RDA). Similarities between the separated fragments and the human genomic DNA were analyzed with Basic Local Alignment Search Tool (BLAST).

RESULTS: Three differentially methylated DNA sequences were obtained, two of which have been accepted by GenBank. The accession numbers are AY887106 and AY887107. AY887107 was highly similar to the 11th exon of LOC440683 (98%), 3' end of LOC440887 (99%), and promoter and exon regions of DRD5 (94%). AY887106 was consistent (98%) with a CpG island in ribosomal RNA isolated from colorectal cancer by Minoru Toyota in 1999.

CONCLUSION: The methylation degree is different between gastric cancer and normal gastric mucosa. The differentially methylated DNA sequences can be isolated effectively by MS-RDA.

INTRODUCTION

Epigenetic inactivation of tumor suppressor genes (TSG) is frequently associated with tumor pathogenesis^[1,2]. The major mechanism of this epigenetic inactivation is through hypermethylation of promoter CpG islands (CGIs). In general, DNA hypermethylation of gene-associated CpG islands results in either down-regulation or complete abrogation of gene expression, indicating that aberrant DNA methylation could execute a similar function to genetic abnormalities, such as inactivating mutations or deletions in the disease state^[1,3,4]. Numerous studies have indicated that several gene classes such as adhesion molecules, inhibitors of angiogenesis, DNA repair, cell cycle regulators, metastasis suppressors, *etc* are frequently hypermethylated in human primary tumors^[5-9]. Gastric cancer is the second most common cause of cancer death in the world^[10]. Compared with other malignant tumors, the incidence and mortality rate of gastric cancer rank first in China^[11,12]. In the present study, DNA methylation is characterized as an important mode of epigenetic modification in the development and progression of gastric cancer by affecting gene transcription, increasing the frequency of gene mutation, and enhancing genomic instability^[13]. However, the entire picture of methylation-silenced genes in gastric cancers is still unclear, and further searching for methylation-silenced genes is necessary.

Because analysis of methylation of known genes has limitations, genome-wide screening for CGIs methylated in gastric cancer is needed. In this study, methylation-sensitive representational difference analysis (MS-RDA)^[14-16], an advanced biotechnique, was used to detect the methylation

status of two genomes and elucidate the role of methylation in carcinogenesis. Moreover, with this strategy, novel genes related to gastric cancer can be obtained and new targets can be offered for gastric cancer research.

MATERIALS AND METHODS

Materials

Tissues from three gastric adenocarcinomas and adjacent normal tissue samples (> 5 cm away from the center of cancer) were collected from gastric cancer patients at a hospital affiliated with Nanhua University. Tumor and adjacent normal tissues were isolated and stored in liquid nitrogen. The histological types were confirmed by histological examination. The study protocol was approved by the Ethics Committee of Nanhua University.

DNA extraction and HpaII digestion

Genomic DNAs of three gastric adenocarcinomas and adjacent normal mucosa samples were prepared by serial extraction with phenol and chloroform, followed by ethanol precipitation. A 10 µg sample of genomic DNA was digested with 10 µL methylation-sensitive four-base recognition restriction enzymes Hpa II (Fermentas Co., 10 U/µL), and was incubated for 20 h at 37°C.

Amplicon preparation

Adaptors RHpa24 and RHpa11 (Table 1) were ligated to the mixture of digestion products of three gastric adenocarcinomas and adjacent normal mucosa samples, annealed by gradual cooling from 50°C to 10°C for 40 min, and then ligated to DNA fragments by overnight incubation with T4 DNA ligase at 16°C^[17]. The ligation product was amplified for 26 cycles (each cycle including 1 min incubation at 95°C and 3 min at 72°C, with the last cycle followed by an extension at 72°C for 10 min) with RHpa24 oligonucleotides as a primer as reported. Three gastric adenocarcinoma samples served as the testers, and three adjacent normal mucosa samples served as the drivers. The DNA fragments with RHpa adaptor were amplified effectively and testers and driver amplicons were obtained^[14]. Amplicons were detected by 1.0% agarose gel electrophoresis.

Subtractive hybridizations

The RHpa adaptor in the tester and driver amplicons were removed by digestion with the isoschizomerase MspI (Fermentas Co. 10 U/µg) and separated with a DNA fragment purification and filtration kit (Takara Co., Japan). The JHpa II_{24/11} adaptor was then ligated to the tester amplicon with T4 DNA ligase. The subtractive hybridizations with gastric cancer amplicons serving as the tester and normal gastric mucosa amplicons serving as the driver were presumed to be the positive hybridizations, and the converse was presumed to be the reverse hybridizations. The tester DNA with J adaptor at its ends was mixed with the driver DNA at a ratio of 1:40. The DNA mixture was purified by phenol extraction and ethanol precipitation, dissolved in 4 µL of 3 × EE buffer (3 mmol/L EDTA/3 mmol/L N-[2-hydroxyethyl] piperazine-N'-[3-propansulfonic acid], pH 8.0), denatured at 96°C for 10 min, and reannealed

Table 1 Adaptors and sequences in MS-RDA

Primer	Sequences
RHpa II ₂₄	5'-AGC ACT CTC CAG CCT CTC ACC GAC-3'
RHpa II ₁₁	5'-CGG TCG GTG AG-3'
JHpa II ₂₄	5'-ACC GAC GTC GAC TAT CCA TGA AAC-3'
JHpa II ₁₁	5'-CGG TTT CAT GG-3'
NHpa II ₂₄	5'-AGG CAA CTG TGC TAT CCG AGG GAC-3'
NHpa II ₁₁	5'-CGG TCC CTC GG-3'
SHpa II ₂₄	5'-ACT TCT ACG GCT GAA TTC CGC CAC-3'
SHpa II ₁₁	5'-CGG TGT CGG AAT-3'

MS-RDA: Methylation-sensitive representational difference analysis.

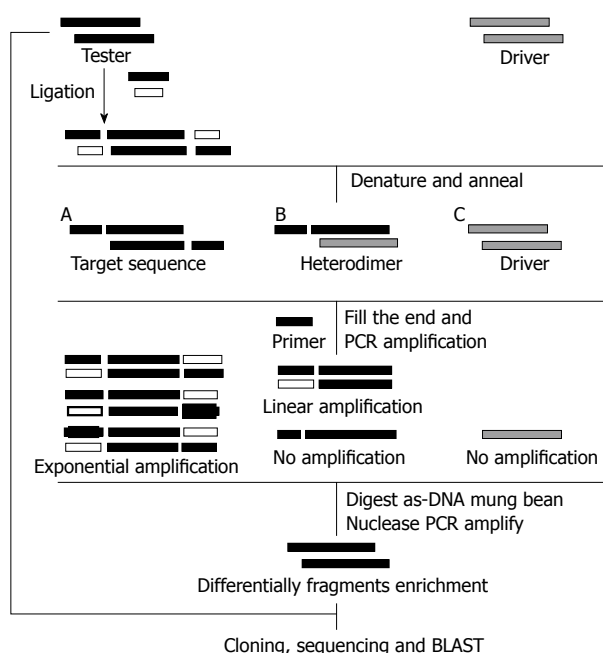


Figure 1 Schematic diagram of MS-RDA experimental strategy.

at 67°C for 24 h. One-tenth of the reannealed product was amplified by PCR with JHpa₂₄ oligonucleotide as the primer for 13 cycles. Tester/tester and tester/driver double-stranded DNA fragments had J adaptors on either both ends or only one end, respectively, and could be amplified exponentially or linearly. DNA fragments linearly amplified, existing as single-stranded DNA, were digested with mung-bean nuclease (Takara Co., Japan), and the remaining double-stranded DNA was again amplified by PCR for 26 cycles with JHpa₂₄^[14].

For the second and third cycles of subtractive hybridization to a new adaptor (N/S adaptor for the second cycle; J for the third cycle) (Table 1), the ratio of the tester DNA to the driver DNA was 1:400 and 1:4000, respectively^[18], and the differentially methylated sequence between gastric cancer and normal gastric mucosa could be obtained. The experiment fluidogram is displayed in Figure 1.

Cloning, sequencing and BLAST

After the final subtractive hybridization, products were electrophoresed through 1.0% agarose gel, and the target bands were recovered with an agarose gel recovery kit

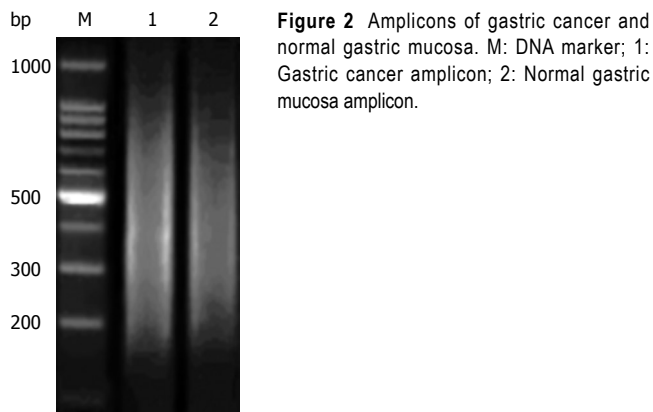


Figure 2 Amplicons of gastric cancer and normal gastric mucosa. M: DNA marker; 1: Gastric cancer amplicon; 2: Normal gastric mucosa amplicon.

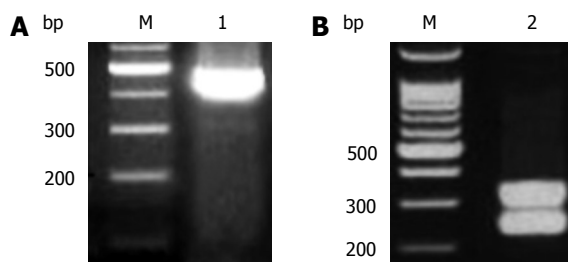


Figure 3 A: Results after the third cycle of competitive hybridization. B: After a second cycle of competitive hybridization. M: DNA marker; 1: differentially methylated fragment of approximately 400 bp; 2: Two differentially methylated fragments, one is approximately 400 bp and the other lies between 200-300 bp.

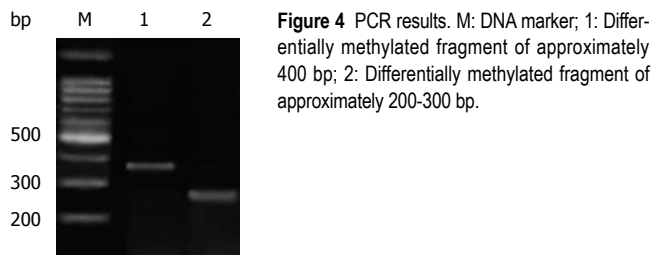


Figure 4 PCR results. M: DNA marker; 1: Differentially methylated fragment of approximately 400 bp; 2: Differentially methylated fragment of approximately 200-300 bp.

(Takara Co., Japan). The target fragments were subsequently cloned into a pGEM-T vector (Promega Co., USA). Plasmid DNA was transformed into *E. coli* strain JM109. Bacteria were incubated in LB medium for 1 h at 37°C, then plated onto agar plates containing ampicillin (100 µg/mL), X-gal (20 µg/cm²) and IPTG (12.1 µg/cm²) and incubated for 14 h at 37°C. Positive (white) colonies were picked out and identified by PCR. The primer was the JHpa24 oligonucleotide. After the positive colonies were sequenced, nucleic acid sequence homology searches were performed using the BLAST server at the National Center for Biotechnology Information.

RESULTS

Amplicons of gastric cancer and normal gastric mucosa

The digestion products of gastric cancer and normal gastric mucosa genomic DNAs were ligated with the RHPa II adaptor using PCR, and the amplicons were enriched (Figure 2). As shown in the figure, amplicons appeared as bright smears between 200-1000 bp.

Differentially methylated DNA fragments

After three cycles of the positive hybridizations, a differentially methylated fragment of about 400 bp could be seen (Figure 3A). After two cycles of the reverse hybridizations, two differentially methylated fragments could be obtained (Figure 3B), one at approximately 400 bp and the other located between 200-300 bp.

Cloning, sequencing and BLAST

Three differentially methylated DNA fragments were cloned into pGEM-T, and the positive colonies were identified by PCR (Figure 4) and sequenced (Figure 5). The differentially methylated DNA fragment from the positive hybridizations was problematic because of its complex secondary structure. Two differentially methylated DNA fragments from the reverse hybridizations were accepted by GenBank. The accession numbers were AY887106 and AY887107, respectively. The two differentially methylated DNA fragments were: AY887106: CCGGCGCCTAGCAGCCGACTTANAACGTG-TGTCGGACCAGGGGAATCCGACTGTTAAT-TAAAACAAAGCATCGCGAAGGCCCGCGGC-GGGTGTGACGCGATGTGATTTCTGCCAGT-GCTCTGGATGTCAAAGTGAAGAAATTCAAT-GAAGCGCGGGTAAACGGCGGGAGTAACCAT-GACTCTCTTAAGGTAGCCAAATGCCTCGTCATC-TAATTAGTGACGCGCATGAATGGATGAACGAGA-TTCCCCCTGTCCCTACCTACTATCCAGCGAAAC-CACAGCCAAGGGAACGGGCTTGCGCGGAAT-CAGCGGGGAAAGAAGACCCTGTTGAGCTT-GACTCTAGTCTGGCACGGTGAAGAGACAT-GAGAGGTGTAGAATAAGTGGGAGGCTCCCGG; AY887107: ATACCAGCAGCTGGCGCAGGGGAAT-GCCGTGGGGGGGCTCGGGCGGGGGCACC GC-CACTGGGGGCCCGTGCAGGTGGTCACCGCCT-GCCTGCTGACCCTACTCGTCATCTGGACCTT-GCTGGGCAACGTGCTGGTGTCCGCAGCCATC-GTGTGGAGCCGCCACCTGCGCGCCAAGAT-GACCAACGTCTTCATCGTGTCTCTACCTGTGT-CAGACCTCTTCGTGGCGCTGCTGGTCATGTCTT-GGAAGGCAGTCGCCGAGGTGGCCGG.

BLAST indicated that AY887107 was highly similar to the 11th exon of gene LOC440683 located on chromosome 1q21.1, while the 3' end of gene LOC440887 located on chromosome 2p11.1 included the promoter and 1st exon region of gene DRD5 located on chromosome 4p16.1, respectively. Their similarity rates were 98%, 99% and 94% respectively. AY887106 located on ribosomal RNA was linked with a CpG island isolated from colorectal cancer by Toyota *et al.*^[19]. The characteristics of differentially methylated DNA fragments are shown in Table 2.

DISCUSSION

Given the role of aberrant DNA methylation in cancer initiation and progression, a distinct effort has been put towards the development of strategies that could facilitate early cancer detection. It is clear that aberrant DNA methylation is an early event in tumor development, as indicated by reports where aberrantly hypermethylated sites could be detected in seemingly normal epithelia from

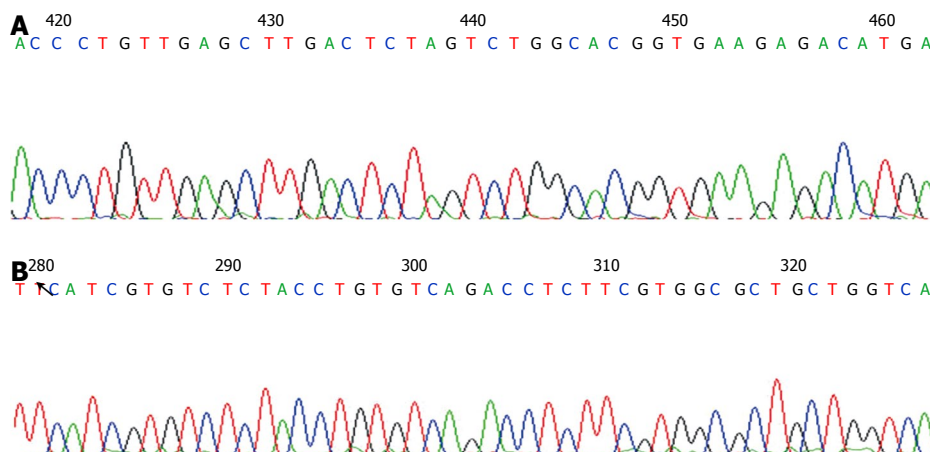


Figure 5 AY887106 (A) and AY887107 (B) sequencing.

Table 2 Characteristics of differentially methylated DNA fragments by MS-RDA

Accession number	Screening time	Size (bp)	GC (%)	CG/GC	Similarity			
					Location	S	E	Similarity rate (%)
AY887106	2	395	50.6	0.9286	8q13.3	745	0	98
AY887107	2	264	66.3	0.7000	2p11.1	521	e-145	99
					1q21.1	498	e-138	98
					4p16.1	435	e-119	94

patients, years before the overt development of cancer^[20]. Thus, utilizing DNA methylation as a biomarker might prove to be a useful tool not only for early diagnosis but also for the detection and assessment of high-risk individuals^[21-23]. This strategy is reported to be successful in various bodily fluids, biopsy materials, lymph nodes obtained at surgery, and serum.

Although Southern Blotting and Restriction Landmark Genomic Scanning have been used for detection of abnormal DNA methylation, it is limited to the detection of known genes and is a complicated and difficult procedure^[24]. Lisitsyn *et al*^[17] established a representational difference analysis (RDA) based upon subtractive hybridization in 1993. For this, two genomic DNAs were digested with sensitive restriction enzyme in order to reduce the structural complexity of genomic DNA. After several cycles of subtractive hybridizations and PCR, the target fragments differentially isolated from two genome specimens were obtained. Subsequently, Ushijima *et al*^[14] developed MS-RDA technology and successfully isolated the differentially methylated genomic fragments in mouse liver tumor. MS-RDA uses the methylation-sensitive four-base recognition restriction enzyme HpaII to digest two genomic DNAs in order to reduce the methylation site complexity of the DNA. The digestion product was ligated to the RHPaII^{24/11} adaptor, and PCR was used to effectively amplify the shorter fragments, while longer fragment amplification was difficult by PCR. Accordingly, the difference between “driver” and “tester” can be transformed to the distinction of different digestion sites^[17]. After several cycles of subtractive hybridizations and PCR, the differentially methylated fragments form homopolymers and are amplified exponentially, while the

identical sequences between “driver” and “tester” form heterodimers and are amplified linearly. Thus, the identical sequences will be eliminated, while the target fragments, existing in the “tester” but lacking in the “driver”, have been amplified effectively. As for the specificity of MS-RDA, it was shown that the contrast between “tester” and “driver” is not a difference in digestion fragment size but rather a difference in the methylation status of two genomes. MS-RDA is an effective method for identifying the silenced genes in various cancers and marker genes linked with diseases.

In this study, MS-RDA was performed using a mixture of DNAs from mucosa of three gastric adenocarcinomas serving as the tester and adjacent normal mucosa serving as the driver. This strategy might have neglected infrequent methylations that occurred in only one gastric adenocarcinoma or that were induced by individual differences. Our data indicate that gene LOC440887 and LOC440683 were highly homologous to the sequences AY887107. Using GenBank, we found that gene LOC440887 and gene LOC440683 are novel genes that are seldom studied. LOC440887 is probably linked to the etiology of myeloid/lymphoid or mixed-lineage leukemia and closely related to the MLL3 gene, which is located on chromosome region 7q36 and encodes a protein homologous to ALR (acute lymphoblastic leukemia-1 related). A previous study by Tan *et al*^[25] demonstrated that MLL3 does map to 7q36, a chromosome region that is frequently deleted in myeloid leukemia and may be involved in leukemogenesis. Ruault *et al*^[26] found partial duplications of the MLL3 gene in the juxtacentromeric region of chromosomes 1, 2, 13, and 21. The LOC440887 gene may be a duplicated region containing a fragment of the MLL3 gene on chromosome 2. Digital Northern analysis was applied to the MLL3 gene using the NCBI Web Station. The outcome indicated that the human stomach tissue could express the MLL3 gene and that there were expression differences between the gastric cancer and the normal stomach tissue. We are currently investigating the relative differences in expression level between the tissue samples. Moreover, BLAST indicated that AY887107 was highly similar to the promoter and 1st exon region of gene DRD5 located on chromosome 4p16.1, with a similarity rate of 94%. The DRD5 gene encodes the D5

subtype of the dopamine receptor. The D5 subtype is a G-protein coupled receptor which stimulates adenylyl cyclase. In both gastric and duodenal mucosa, Mezey *et al*^[27] demonstrated the presence of significant amounts of the D5 receptor that could serve as a target for locally produced dopamine. Dopamine is a protective agent in the gastrointestinal (GI) tract in both rats and humans. The relationship between DRD5 gene methylation and gastric cancer still needs further studies. The sequence AY887106, which maps to ribosome RNA, has 98% homology to a differentially methylated CpG island genomic sequence obtained from human colon carcinomas by Toyota *et al*^[19]. Our research also suggested that this location is likely to be methylated in gastrointestinal carcinomas. In view of the above-mentioned analysis, we will carry out a relative study of three differentially methylated DNA fragments so that novel genes related to gastric cancer can be obtained and new targets can be offered for gastric cancer research.

Various methylation-based strategies, including MS-RDA, restriction landmark genome scanning^[24,28], arbitrarily primed PCR^[29], and CpG island microarray^[30], have been developed and proven to be useful for identifying hypermethylated sequences. MS-RDA was previously established to detect differences in the methylation status of two genomes. As for the specificity of MS-RDA, it was shown that DNA fragments, that are unmethylated in the tester and almost completely methylated in the driver, are efficiently isolated. This indicated that genes that are in a biallelic methylation state or in monoallelic methylation with loss of the other allele are efficiently isolated^[31]. Takai *et al*^[32] first reported the silencing of HTR1B and reduced expression of EDN1 in human lung cancers using MS-RDA. Subsequently, Miyamoto *et al*^[33] found methylation-associated silencing of heparan sulfate D-glucosaminyl 3-O-sulfotransferase-2 (3-OST-2) in human breast, colon, lung, and pancreatic cancers. Then in 2004, Hagihara *et al*^[34] documented that MS-RDA was used for isolation of 111 DNA fragments derived from CpG islands (CGIs), and 35 of them were from CGIs in the 5' regions of known genes in human pancreatic cancers. MS-RDA is effective in identifying silenced genes in various cancers^[35]. MS-RDA also has some disadvantages such as complexity of the procedure, requiring high homology between the "tester and driver", inefficiency with amplicons less than 1 kb. In a word, MS-RDA is a promising method for the study of DNA methylation in gastric cancer. It may also be used for isolating novel tumor suppression genes.

COMMENTS

Background

Epigenetic inactivation of tumor suppressor genes (TSG) is frequently associated with tumor pathogenesis. The purpose of this study was to isolate the differentially methylated DNA sequences between gastric cancer and normal gastric mucosa.

Research frontiers

Genome-wide screening for CGIs differentially methylated between gastric cancer and normal gastric mucosa.

Innovations and breakthroughs

Because analysis of methylation of known genes has limitations, genome-wide screening for CGIs methylated in gastric cancer is needed. In this study,

methylation-sensitive representational difference analysis (MS-RDA), an advanced biotechnique, was used to detect the methylation status of gastric cancer and normal gastric mucosa.

Applications

This study found that methylation status is different between gastric cancers and normal gastric mucosa. It elucidated the role of methylation in carcinogenesis, and moreover, new targets were offered for gastric cancer research.

Terminology

Methylation is a term used in chemical sciences to denote the attachment or substitution of a methyl group on various substrates. DNA methylation profiling is gaining momentum as an epigenetic approach to understanding the effects of aberrant methylation (either hyper- or hypomethylation) both in basic research and in clinical applications.

Peer review

The manuscript by Liao *et al* describes a method to screen differences in methylation status of DNA between gastric cancer samples and normal gastric mucosa. The authors found 3 DNA regions that have changed methylation status between the normal and neoplastic tissue. The data presented is novel and interesting.

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Increased hepcidin expression in colorectal carcinogenesis

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Abstract

AIM: To investigate whether the iron stores regulator hepcidin is implicated in colon cancer-associated anaemia and whether it might have a role in colorectal carcinogenesis.

METHODS: Mass spectrometry (MALDI-TOF MS and SELDI-TOF MS) was employed to measure hepcidin in urine collected from 56 patients with colorectal cancer. Quantitative Real Time RT-PCR was utilized to determine hepcidin mRNA expression in colorectal cancer tissue. Hepcidin cellular localization was determined using immunohistochemistry.

RESULTS: We demonstrate that whilst urinary hepcidin expression was not correlated with anaemia it was positively associated with increasing T-stage of colorectal cancer ($P < 0.05$). Furthermore, we report that hepcidin mRNA is expressed in 34% of colorectal cancer tissue specimens and was correlated with ferroportin repression. This was supported by hepcidin immunoreactivity in colorectal cancer tissue.

CONCLUSION: We demonstrate that systemic hepcidin expression is unlikely to be the cause of the systemic anaemia associated with colorectal cancer. However, we demonstrate for the first time that hepcidin is expressed by colorectal cancer tissue and that this may represent a novel oncogenic signalling mechanism.

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Key words: Iron; Hepcidin; Colon; Cancer; Anaemia;

Mass spectrometry

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INTRODUCTION

Anaemia is a common presenting symptom of colorectal cancer, a recent study showing that up to 6% of patients presenting with iron deficiency anaemia (IDA) had colorectal cancer^[1]. IDA is commoner in patients with right sided than left sided colorectal cancers^[2]. The reason for this is assumed to be because of the chronic occult blood loss associated with cancers in the right colon compared to overt bleeding of left sided colonic disease which is detected sooner.

What remains unclear is whether a component of the anaemia might be a consequence of the inflammation associated with the neoplastic process. The major pro-inflammatory cytokine implicated in the pathogenesis of anaemia of chronic inflammation is interleukin 6 (IL-6). Recent work has shown that the induction of hepcidin release stimulated by IL-6 is crucial to this process^[3-5]. Precisely how hepcidin mediates anaemia of chronic disease has been comprehensively investigated by several groups and it is now well established that hepcidin acts to cause a block on cellular iron export by internalization and degradation of ferroportin^[6,7]. At the level of the macrophage, the major cell type responsible for iron recycling, this results in iron sequestration and interrupts iron delivery to erythroid precursor cells thus causing anaemia^[8]. Increased hepcidin levels are likely to also cause an accumulation of iron in other ferroportin expressing cell lineages such as colonocytes. Our recent studies have shown that raising colonocyte iron levels can result in increased Wnt signalling which has been shown to be crucial in colorectal carcinogenesis^[9,10].

However, to date there are no studies addressing whether hepcidin expression contributes to the anaemia in colorectal cancer and whether hepcidin itself may be considered a pro-oncogenic factor.

There are several strands of evidence to suggest that this hypothesis may be relevant: firstly, the major inducer of hepcidin, IL-6, shows increased expression in both colorectal cancer tissue and in the serum of colorectal cancer patients and is indicative of a more advanced phenotype^[11-13]. Secondly, consistent with a hepcidin-colonocyte axis, we have reported that ferroportin is cytoplasmic in cellular localization and expression levels are repressed with increasing stage of colorectal cancer^[14].

This raises the possibility that hepcidin may have a dual effect in colorectal cancer patients; contributing to the systemic anaemia by acting at the level of the macrophage whilst acting locally at the colonocyte level promoting iron accumulation and Wnt signalling.

Thus the aims of this study were: (1) To measure urinary hepcidin levels by Matrix-assisted laser desorption/ionization time of flight mass spectroscopy (MALDI-TOF MS) and Surface-enhanced laser desorption ionization time of flight (SELDI-TOF MS) in colorectal cancer patients and determine if hepcidin expression was associated either with anaemia or with disease state including T-stage, and nodal involvement; (2) To examine if hepcidin mRNA expression could also be detected in colorectal cancer tissue and, if so, determine cellular localization.

MATERIALS AND METHODS

Sample collection

Urine was collected from 56 patients with colorectal cancer prior to surgery, centrifuged at 3000 r/min for 20 min and stored at -80°C. In a separate set of colorectal cancer patients ($n = 34$) surgical resection specimens of colorectal carcinoma matched with normal colonic mucosa from the same resection specimen were collected and processed for RNA extraction.

Ethics

This work has been carried out in accordance with the declaration of Helsinki (2000) of the World Medical Association. Ethical approval for this study was approved by South Birmingham LREC No. 05/Q2702/17. All patients provided informed written consent.

Urine hepcidin measurements

SELDI-TOF-MS was performed as previously described^[15]. Briefly, urine samples were diluted to 10 µg protein/mL in 0.5 mol/L NaCl, 100 mmol/L sodium phosphate (pH 7.0) and applied to Cu²⁺ loaded IMAC30 ProteinChip arrays. MALDI spectra were obtained either by applying diluted urine (20 µg protein/mL) or urine desalted using ClinProt C8 magnetic beads (BrukerDaltronic) to GoldChips. Spectra were acquired on a PBS II c ProteinChip Reader (Ciphergen) using sinapinic acid as the matrix. Spectra were normalized to total ion current (as previously described for urinary biomarker profiling^[16,17]), baselines subtracted and peaks picked using Ciphergen ProteinChip software. Synthetic hepcidin-25 (Peptides International) was employed for peak/assay validation. Immunocapture was performed as previously described^[15,19]. Briefly Protein G sepharose beads loaded with or without rabbit polyclonal anti-hepcidin-25

(Abcam 31877) were incubated with human urine containing hepcidin 20, 22 and 25. The beads were washed extensively with 20 mmol/L ammonium bicarbonate and the captured proteins eluted with 50% acetonitrile/0.1% trifluoroacetic acid and analysed by MALDI.

Real time RT-PCR

Real Time RT-PCR was performed on colorectal cancer tissue specimens (C) and matched uninvolved normal colonic mucosa (N) with all reactions containing 18S ribosomal RNA as an internal standard (PE Biosystems, Roche, USA), and human specific hepcidin probe and primers (Probe 5'-FAM 3' TAMRA AGCTGCAACCCCAGG, Forward primer CCCACAACAGACGGGACAA and Reverse primer TCTGGAACATGGGCATCC) as previously described^[18].

Immunohistochemistry

Immunohistochemistry was performed using paraffin sections of human normal colon ($n = 10$) and colorectal cancer ($n = 15$). All sections were incubated at 95-100°C in WCAP reagent (Surgipath) for 30 min, washed and then incubated in 10:1 Methanol: H₂O₂ for 10 min. Sections were then incubated in rabbit polyclonal hepcidin antibody (Abcam 31877; 1:20) for 1hr after which immunoreactivity detected with an Envision assay kit and DAB reagent (DakoCytomation). All sections were counterstained in haematoxylin and mounted prior to visualisation. Normal liver sections were utilized as a positive control. Negative control included omission of primary antibody and liver sections incubated with antibody which had prior incubation with the immunizing peptide (Abcam 31875).

Statistical analysis

Statistical significance was calculated using the Mann-Whitney test to compare continuous data and the Spearman rank test to assess correlations between data sets. Significance was accepted at $P \leq 0.05$. All analyses were performed using SPSS version 14.0 (SPSS Inc, USA). Data are presented with 2 standard errors of the mean.

RESULTS

Validation of MALDI-TOF MS for the detection of urinary hepcidin

Using a pre determined hepcidin positive urine sample we performed MALDI-TOF MS and were able to detect the two major forms of hepcidin; the mature hepcidin 25 (m/z 2793.8), and the N-terminally truncated hepcidin 20 (m/z 2195.3). In addition we were also able to detect hepcidin 22 (m/z 2440.5) which corresponds to a urinary degradation product of hepcidin 25 (Figure 1Aa). Using a human urine sample devoid of hepcidin (Figure 1Ab) we were able to demonstrate the appearance of a hepcidin 25 peak on spiking with a synthetic human hepcidin peptide (Figure 1Ac). To determine if MALDI-TOF MS could be used in a semi-quantitative manner as has previously been reported for SELDI-TOF MS we spiked a urine sample with low endogenous hepcidin and demonstrated a linear relationship between hepcidin concentration and intensity of the hepcidin 25 peak (Figure 1B).

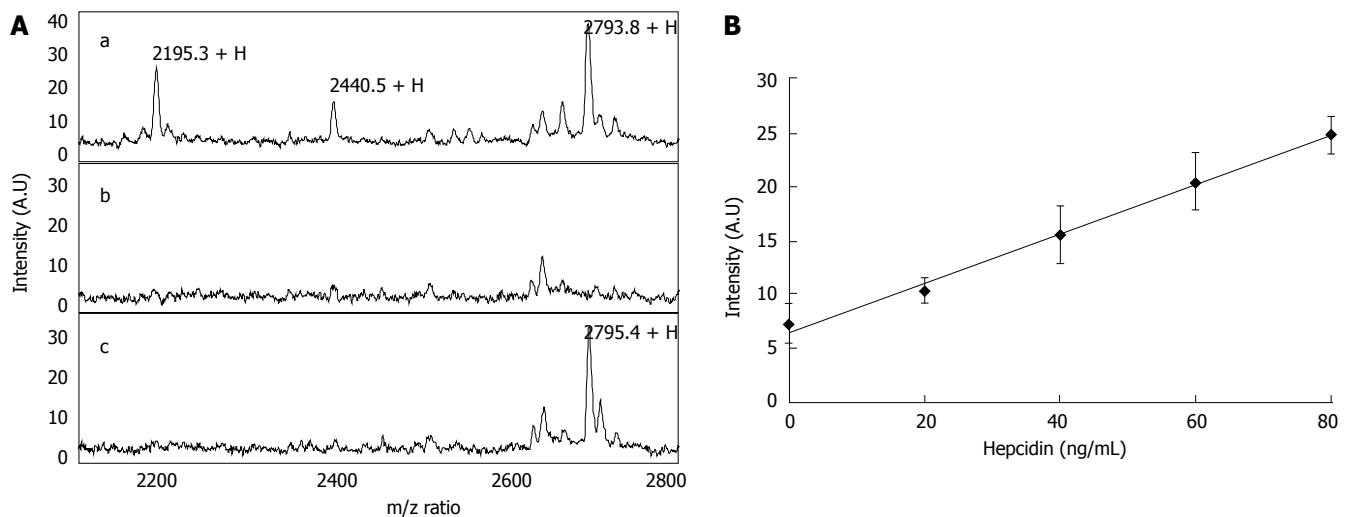


Figure 1 Validation of hepcidin expression in urine by MALDI-TOF MS. **A:** (a) A human urine specimen showing the two dominant forms of hepcidin; hepcidin 20 (m/z 2195.3) and hepcidin 25 (m/z 2793.8). In addition the degradation product of hepcidin 25 hepcidin 22 could also be detected by MALDI-TOF MS (m/z 2440.5); (b) A human urine specimen completely devoid of hepcidin which when spiked with synthetic hepcidin 25 clearly shows a detectable peak at 2795.4 (c); **B:** Synthetic hepcidin was spiked into a low hepcidin containing urine sample at concentrations between 0-80 ng/mL and analysed by MALDI-TOF MS followed by analysis of the hepcidin 25 peak intensity.

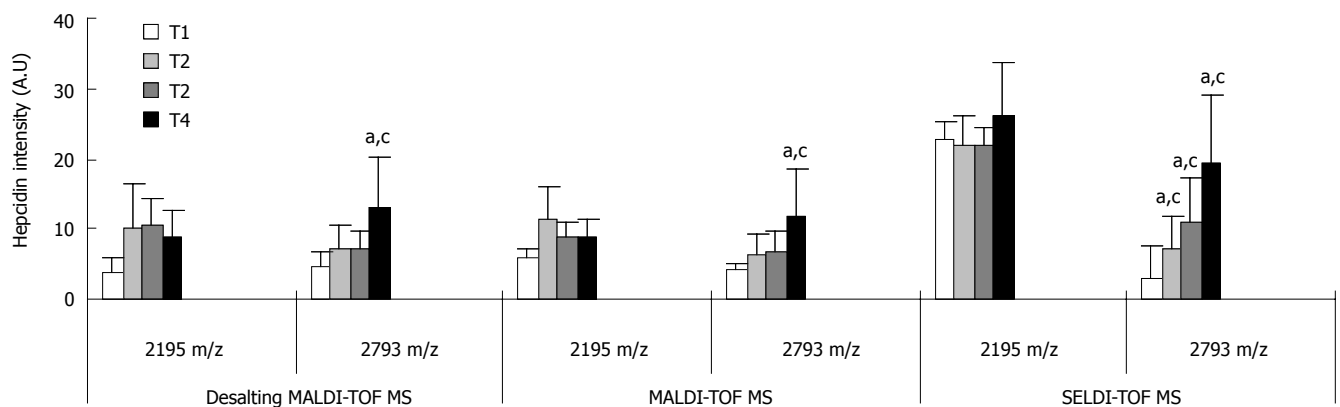


Figure 2 Hepcidin expression is associated with T-stage of colorectal cancer. Expression of hepcidin 20 (m/z 2195) and 25 (m/z 2793) was determined in urine samples of 56 colorectal cancer patients by MALDI-TOF MS, before and after desalting with desalting beads (Desalting MALDI-TOF MS), and SELDI-TOF MS and analysed with respect to T-stage. Expression of hepcidin 20 was not altered with T-stage by any of the three techniques. However, all three techniques demonstrated a significant increase in hepcidin 25 expression in T4 cancers compared to T1. Statistical significance compared to T1 ($^*P < 0.05$); Statistical significance compared to preceding T-stage ($^cP < 0.05$). Error bars denote 2 SEM.

Analysis of urinary hepcidin levels in colorectal cancer patients

To determine hepcidin levels in urine samples from colorectal cancer patients ($n = 56$) we performed SELDI-TOF MS and MALDI-TOF MS (Figure 2). In addition, as the presence of salt can interfere with mass spectrometry, we desalted the urine samples and once more performed MALDI-TOF MS. Since hepcidin 22 is a urine specific degradation product of hepcidin^[19] we chose to analyse only the expression level of hepcidin 20 and 25. Using a Spearman Rank test we were able to demonstrate a correlation between all three data sets (MALDI-TOF MS *vs* SELDI-TOF MS, correlation coefficient = 0.59 $P < 0.0001$, Desalting MALDI-TOF MS *vs* MALDI-TOF MS, correlation coefficient = 0.56 $P < 0.0001$, Desalting MALDI-TOF MS *vs* SELDI-TOF MS, correlation coefficient = 0.71 $P < 0.0001$).

Data was analysed with respect to the site of cancer

(left or right sided), TNM classification, and the presence or absence of anaemia where anaemia was classified as haemoglobin level of less than 13 g/dL for men and 12 g/dL for women. Of the 56 cancers examined, 63% were left sided cancers of which 40% were anaemic, whereas of the right sided cancers 77% were anaemic^[22]. Our data analyses using all data sets individually (Desalting MALDI-TOF MS, MALDI-TOF MS, and SELDI-TOF MS) for both hepcidin 20 and 25 showed that neither forms of hepcidin were significantly altered in expression in respect to the site of cancer, local nodal involvement, metastasis or haemoglobin level.

However, when examining the relationship between hepcidin and T-stage of disease, whilst hepcidin 20 was not correlated with T-stage, hepcidin 25 significantly increased with increasing stage. All three mass spectrometry techniques demonstrated a significant increase in hepcidin 25 in T4 cancers compared to T1. Moreover, SELDI-TOF

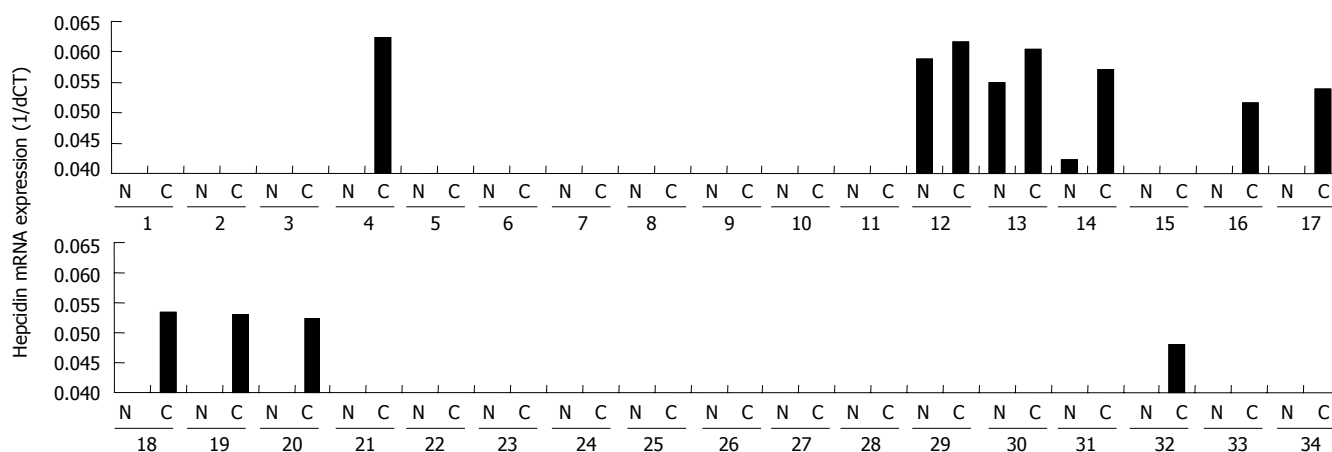


Figure 3 Hepcidin mRNA expression in colorectal cancer tissue. Using Real Time RT-PCR on 34 cancer specimens (C) each with adjacent normal uninvolvement mucosa (N) we demonstrate that hepcidin mRNA expression could be detected in 34% of colorectal cancer tissue specimens (10 out of 34). mRNA expression is presented as 1/dCT. Positive control included human liver. Negative control included omission of cDNA.

MS data delineated a significant stepwise increase between T2 and T1 (7.28 *vs* 2.77 $P < 0.05$), T3 and T2 (11.07 *vs* 7.28 $P < 0.05$), and T4 and T3 (19.17 *vs* 11.07 $P < 0.05$).

Determination of hepcidin expression and localization in colorectal cancer tissue

The observation that hepcidin is associated with advanced disease led us to speculate that it may have an effect at the level of the colonocyte and that hepcidin may even be expressed by colorectal cancer tissue. Thus we examined hepcidin mRNA expression in 34 cancer specimens (C) each with adjacent normal uninvolvement mucosa (N) (Figure 3). Our data shows that hepcidin mRNA was detected in 34% of colorectal cancer tissue specimens (10 out of 34) and, whilst there was no hepcidin mRNA detectable in the majority of matched adjacent uninvolvement mucosa, where there was hepcidin mRNA, it was found at a lower level than in the associated cancer. Furthermore we find that hepcidin mRNA expression is inversely correlated with ferroportin mRNA expression (data not shown).

To further verify expression and determine the cellular localization of hepcidin we performed immunohistochemistry. As a positive control for hepcidin expression we utilized human liver. As anticipated we were able to show strong immunoreactivity in all hepatocytes (Figure 4Aa), and this immunoreactivity could be blocked by co-incubation with hepcidin peptide (Figure 4Ab). In normal colon, both on the luminal surface and in the crypts, we were unable to detect any hepcidin immunoreactivity (Figure 4Ac-Ad). However, in approximately 46% (7 out of 15) of colorectal cancer specimens examined there was an abundance of both membranous (Figure 4Ae-Af) and cytoplasmic immunoreactivity (Figure 4Ag-Ah).

To determine what form of hepcidin was being detected by our antibody and thus expressed in colorectal cancer tissue, we performed an immuno-capture experiment on human urine followed by MALDI-TOF MS (Figure 4B). We initially chose a urine sample which had clear and abundant hepcidin 20, 22 and 25 peaks (Figure 4Ba), and incubated with either protein G sepharose or with protein G-sepharose and the polyclonal anti

hepcidin antibody. Immunocaptured proteins were then eluted from the protein G sepharose and analysed by MALDI-TOF MS. We clearly demonstrated that incubation with the rabbit hepcidin antibody results in binding of all three forms of hepcidin (Figure 4Bc), whilst incubation with protein G sepharose alone did not bind any form of hepcidin (Figure 4Bb). Thus from this data we infer that the immunoreactivity in colorectal cancer tissue could be due to any of these hepcidin moieties.

DISCUSSION

Anaemia is a common presenting symptom of colorectal cancer, and it is widely assumed to be a consequence of blood loss^[1,2]. However, it is possible that a component of this anaemia may be a result of inflammatory processes. In particular the anti-microbial peptide hepcidin a downstream target of cytokines such as IL-6 has been widely accepted as a mediator of anaemia of inflammation^[3-5]. Thus in this study we aimed to address whether hepcidin was involved in the anaemia associated with colorectal carcinogenesis.

We demonstrated using SELDI-TOF MS; a previously validated method for the detection of urinary hepcidin^[19-21], and MALDI-TOF MS; a more common proteomic technique, that urinary hepcidin 25 expression was not associated with the presence of anaemia in a small cohort of 56 colorectal cancer patients. However, it is important to point out that the only parameter which was utilized to assess anaemia was a haemoglobin level of less than 13 g/dL for men and 12 g/dL for women. Thus it is quite possible that some of these anaemic individuals may actually have anaemia of chronic disease rather than pure iron deficiency anaemia. To unequivocally discriminate between these two types of anaemia it would be essential to assess both the level serum ferritin and soluble transferrin receptor levels.

However, our result is consistent with previous reports which suggest that the phenotype of the systemic anaemia associated with colorectal cancer is more likely to be an iron deficiency rather than anaemia of chronic disease and

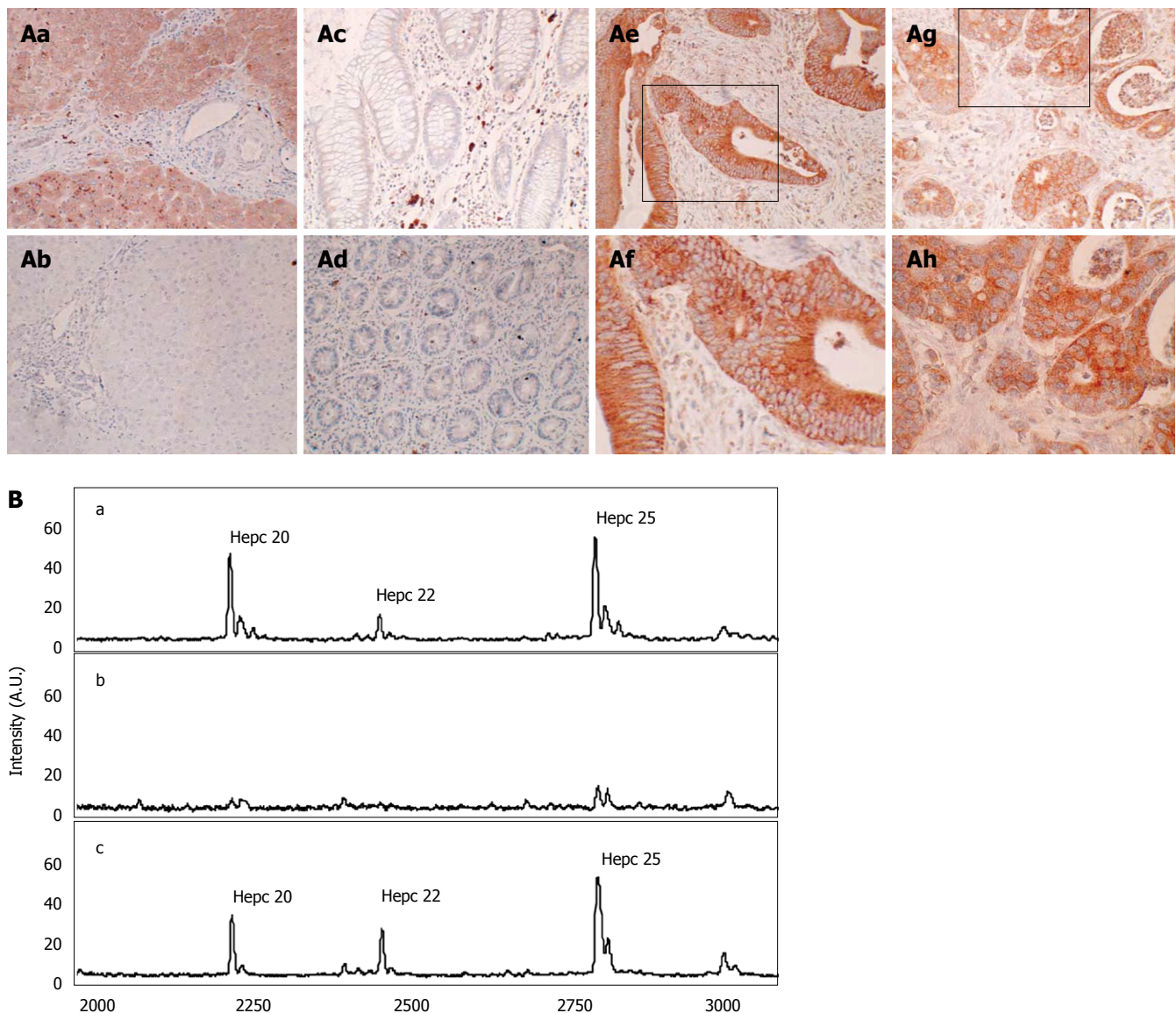


Figure 4 Cellular localization of hepcidin in colorectal cancer tissue. **A:** To determine hepcidin cellular localization in colorectal tissue immunohistochemistry was performed with a hepcidin specific antibody (Abcam 31877). (Aa) Human Liver; (Ab) Human Liver, incubated with both hepcidin antibody and immunizing hepcidin peptide; (Ac) Normal colon; (Ad) Normal colon with crypts in cross section; (Ae) Colorectal cancer (x 20); (Af) Colorectal cancer (x 40); (Ag) colorectal cancer (x 20); (Ah) Colorectal cancer (x 40). Boxes denote area subsequently magnified; **B:** Immunocapture of urinary hepcidin. (Ba) A human urine sample containing hepcidin 20 (m/z 2193.6), 22 (m/z 2438.2) and 25 (m/z 2792.0) was subject to immunocapture with either (Bb) protein G sepharose or with (Bc) protein G-sepharose and a polyclonal anti hepcidin antibody. Immunocaptured proteins were then eluted from the protein G sepharose and analysed by MALDI-TOF MS.

that early detection of blood loss due to colon cancer is life saving^[23].

Interestingly using both MALDI-TOF MS and SELDI-TOF MS we showed that urinary hepcidin 25 expression was associated with stage of disease. This association was not observed for the N-terminally truncated hepcidin 20 moiety. This observation led us to speculate that it may have an effect at the level of the colonocyte and that hepcidin may even be expressed by colorectal cancer tissue. Such a hypothesis is strengthened by evidence that the major inducer of hepcidin, IL-6, is over expressed in colorectal cancer tissue and its expression is positively associated with stage of disease^[11-13]. Moreover, we have recently reported both ferroportin internalization and iron loading in colorectal cancer tissue: both predicted effects of hepcidin^[14]. Thus consistent with such a hypothesis we were

able to demonstrate for the first time that approximately a third of all colorectal cancer tissues examined expressed hepcidin mRNA.

What the signal for hepcidin induction is in these colorectal cancers is unclear. The two original reports describing murine hepcidin suggested that there was either very low or no hepcidin expressed in the colon consistent with our findings^[24,25]. However, more recently it has been suggested that as a consequence of an acute phase response the colon does have the potential to induce hepcidin expression^[26] and furthermore several of the upstream regulators of hepcidin including IL-6, STAT3, Tfr2 and BMP4 have been shown to be over expressed in colorectal cancer tissue^[11-13,27-29]. In addition a recent report suggests that hepcidin expression is dependent on p53 status, a tumour suppressor which is commonly mutated in colorectal cancers^[30].

Irrespective of the mechanism of hepcidin expression the downstream consequences are likely to be internalization and degradation of the cellular iron export protein ferroportin. In the background of an elevation in the cellular iron import proteins such as transferrin receptor 1 and Divalent metal transporter 1, this culminates in cellular iron accumulation; a phenotype which we have previously reported in colorectal cancer^[14]. Furthermore, we have recently demonstrated that elevating intracellular iron in the presence of mutations in either adenomatous polyposis coli (APC) or β -catenin results in increased Wnt signalling; the major oncogenic signalling pathway in the colon^[9,10]. Thus hepcidin expression at the level of the tumour may be a mechanism of ultimately accentuating carcinogenesis and that abrogating hepcidin expression either directly or indirectly through IL-6 may provide a strategy for therapeutic intervention.

COMMENTS

Background

Anaemia is a common presenting symptom of colorectal cancer, though whether the anaemia is a consequence of blood loss is not known. Recent studies have suggested that anaemia in the context of chronic disease is mediated by the antimicrobial peptide hepcidin.

Research frontiers

To date there have been no studies addressing whether hepcidin could be the cause of the anaemia associated with colorectal cancer. Similarly, whether colorectal cancer tissue itself can express hepcidin is unknown.

Innovations and breakthroughs

This is the first study to show that systemic hepcidin levels were positively associated with stage of colorectal cancer. Furthermore, this study suggests that colonic epithelial cancer cells have acquired the ability to express hepcidin; a protein which was previously thought to be largely expressed by the liver.

Applications

What still remains unclear is whether the circulating level of hepcidin is derived from the liver or whether it has come from the colorectal tumour tissue itself. Irrespective of this, this study suggests that circulating hepcidin levels may provide a useful tool for aiding in the diagnosis of colorectal cancer.

Terminology

Hepcidin is an antimicrobial peptide which regulates iron metabolism. In instances of high hepcidin it depresses duodenal iron absorption and sequesters iron in the reticulo-endothelial system culminating in anaemia. This type of anaemia is classified as the anaemia of chronic disease and not iron deficiency anaemia which can result from extensive blood loss, compromised duodenal iron absorption and/or low dietary iron intake.

Peer review

This is the first study to associate hepcidin expression with carcinogenesis.

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VIRAL HEPATITIS

COOH-terminal deletion of HBx gene is a frequent event in HBV-associated hepatocellular carcinoma

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associated HCC tissues in China. HBV integration had also taken place in partial HCC tissues. This supporting the hypothesis that deletion and probably integrated forms of the HBx gene may be implicated in liver carcinogenesis.

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Key words: Hepatitis B virus; X gene; Hepatocellular carcinoma; COOH-terminal deletion mutation; Integration

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Abstract

AIM: To investigate the hepatitis B virus (HBV) x gene (HBx) state in the tissues of HBV-related hepatocellular carcinoma (HCC) in Chinese patients and whether there were particular HBx mutations.

METHODS: HBx gene was amplified and direct sequencing was used in genomic DNA samples from 20 HCC and corresponding non-cancerous liver tissues from HBsAg-positive patients. HBV DNA integration and HBx deleted mutation were validated in 45 HCC patients at different stages by Southern blot analysis and polymerase chain reaction methods.

RESULTS: The frequencies of HBx point mutations were significantly lower in HCC than their corresponding non-cancerous liver tissues (11/19 vs 18/19, $P = 0.019$). In contrast, deletions in HBx gene were significantly higher in HCC than their non-cancerous liver tissues (16/19 vs 4/19, $P < 0.001$). The deletion of HBx COOH-terminal was detected in 14 HCC tissues. A specific integration of HBx at 17p13 locus was also found in 8 of 16 HCC, and all of them also exhibited full-length HBx deletions. Integrated or integrated coexistence with replicated pattern was obtained in 45.5% (20/45) - 56.8% (25/45) tumors and 40.9% (18/45) - 52.3% (23/45) non-tumor tissues.

CONCLUSION: HBx deletion, especially the COOH-terminal deletion of HBx is a frequent event in HBV-

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common cancers worldwide and a leading cause of death in many countries, mainly in Asia and Africa. The most prominent factors associated with HCC include chronic hepatitis B and C viral infection, chronic alcohol consumption, aflatoxin-B1-contaminated food and virtually all cirrhosis-inducing conditions^[1,2]. The hepatitis B virus (HBV) genomic DNA contains four partially overlapping open reading frames: preC/C, P, preS/S and X gene^[3-5]. The X gene product, HBV X protein (HBxAg) is a trans-activating protein, and a multifunctional regulator that modulates transcription, signal transduction, cell cycle progress, protein degradation pathways, apoptosis, and genetic stability by directly or indirectly interacting with host factors^[6,7]. Transgenic mice created with the X gene have been reported to develop HCC^[8-10]. Although the transactivation properties of the X gene and HBxAg overexpressed selectively in human HCC^[11,12], its precise role in human hepatocarcinogenesis is still unclear^[1,2]. In some studies, X gene containing substantial but different deletions in the COOH-terminal region was found in many HBV-infected HCC patients^[13-16]. To further explore the relationship between nucleotide changes in HBx and the development of HCC, we examined the mutants and

nucleotide sequence of the HBx gene obtained from the tissues of HBV-related HCC in Chinese patients.

MATERIALS AND METHODS

Tissue samples

Twenty HCCs and corresponding noncancerous liver tissues were obtained from surgically resected samples at Changhai Hospital between August 2003 to October 2004. Patients had not received any treatment before. This study included 18 men and 2 women. Their ages ranged from 31 to 69 years (median 50 years). All patients were positive for serum hepatitis B surface antigen (HBsAg). The median tumor size was 5.5 cm (2.0-12.5 cm). Histopathological diagnosis was made according to the World Health Organization histological classification of tumors of the liver and intrahepatic bile ducts^[17]. Three HCC were well differentiated, 14 were moderately differentiated, and 3 were poorly differentiated. Of the 20 patients, 6 had evidence of intrahepatic metastasis (portal vein invasion and/or intrahepatic dissemination). Sixteen HCC cases were accompanied with liver cirrhosis and the remaining 4 cases were with chronic hepatitis.

Polymerase chain reaction and sequence analysis of HBX

Genomic DNA was extracted from 20 frozen HCC tissues and corresponding non-cancerous liver tissues using the standard phenol/chloroform extraction and ethanol precipitation method. To amplify the integrated HBx sequences from tissues, we used an HBx-Alu PCR-based approach^[18]. The sequences of the primers were as follows: 5'-TGCCAAGTGTGTTGCTGACGC-3' (HBV 1176-1195, AY220699), 5'-AAGGAAAGAAGTCAG AAGG-3' (HBV 1960-1978)^[7]. The fragment size is 803 bp. After denaturation at 94°C for 2 min, 36 cycles of DNA amplification were performed at 94°C for 30 s, at 53°C for 60 s, and at 72 °C for 60 s; with a final extension at 72°C for 10 min and stored at 4°C. PCR results were identified by electrophoresis on a 1% agarose gel stained with ethidium bromide.

HBx gene sequence was directly determined by automated DNA sequencing (ABI PRISMTM 3730 DNA Sequencer) after purified on silica columns (QIAquick PCR purification kit, Qiagen, Courtaboeuf, France). At least two independent DNA extractions and PCR reactions were performed for each sample, and four sequencing reactions (two for each strand) were carried out to confirm that the reported sequence reflected the most prevalent HBx in a specific sample. The plasmid pHBXB1 containing the full length of the HBV X gene cloned in the pcDNA3 vector, was used as a positive control.

Detection of HBV DNA integration and HBx deleted mutation

To evaluate HBV DNA integration and HBx deleted mutation in HCC tissues, genomic Southern blot analysis and PCR were performed on carcinoma tissues and the corresponding non-cancerous liver tissues from a set of independent samples in 45 HCC patients at different stages from Northwest of China, a relative low-aflatoxins

exposure area. No patient had received treatment before. Serum hepatitis B surface antigen (HBsAg) was positive in all patients. The non-tumor tissues exhibited cirrhosis in 43 patients.

HBV DNA integration by genomic Southern blot analysis

Genomic DNA was extracted from frozen HCC tissues and corresponding non-cancerous liver tissues using the standard phenol/chloroform extraction and ethanol precipitation method. Each sample of extracted genomic DNA (8 µg) was subjected to restriction by EcoRI and Hind III, separated on agarose gel and blotted onto nylon membrane (Zetabind, Life Sci., Ltd.). Southern blot analysis was carried out using 32P dCTP DNA Labeling and Detection Kit (Amersham Life Sci., Ltd., England) with the 32P dCTP-labeled HBV, HBc, HBx coding region and Pre-S as probes (Dupont).

Polymerase chain reaction

HBc, HBc and HBx genes were amplified from HCC genomic DNA by polymerase chain reaction (PCR). The sequences of those primers were HBc: P1 (1903-1929), 5'-ATGGACATCGACCCTTATAAAGAATTG-3', P2 (2434-2411): 5'-CTAAGATTGAGATCTTCTGAGAC GCGG-3'; HBc and HBx: P2 (2434-2411): 5'-CTAAGA TTGAGATCTTCTGAGACGCGG-3'; P3 (1227-1243): 5'-AGCGCATGCGTGGAACC-3'. The fragment sizes are 531 bp and 1207 bp, respectively. All PCRs were performed using a Thermal Cycler 9600 (Perkin Elmer, CA) under the following conditions: after denaturation at 94°C for 2 min, 38 cycles of DNA amplification was performed at 94°C for 60 s, at 55°C for 60 s, and at 72°C for 120 s; with a final extension at 72°C for 10 min and stored at 4°C. PCR results were analyzed by electrophoresis on a 1% agarose gel stained with ethidium bromide. To assess the specificity of our results, Southern blot analysis was carried out as described above.

Statistical analysis

All statistical analyses were carried out using the SPSS.11 software. Comparisons between two characteristics were made using Chi-square test or Fisher's exact test. $P < 0.05$ was considered statistically significant in a two-tail analysis.

RESULTS

HBx gene sequencing in HBsAg-positive HCC tissues

To confirm the HBx deletion mutation, HBx gene was amplified and direct sequencing was made in 20 HBsAg-positive HCC genomic DNA. The fragment sizes of PCR products ranged from 150 bp to 800 bp (Figure 1). The results of sequencing were compared with the known sequences in the GenBank database using the BLAST programs (Figure 2). The frequencies of HBx point mutations were significantly lower in HCC than in the corresponding non-cancerous liver tissues (11/19 *vs* 18/19, $P = 0.019$). In 19 available non-tumorous livers, sequencing results revealed 47 different point mutation patterns, including 30 mis-sense mutations, 15 sense mutations and 2 non-sense mutations. The loci with the highest frequency

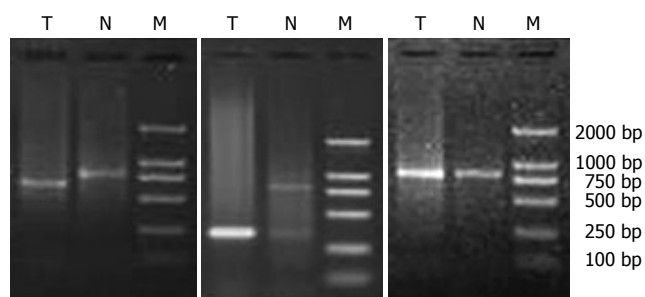


Figure 1 PCR amplified products of HBx-DNA. The fragment sizes of PCR products range from 150 bp to 800 bp. M: Marker; T: Tumor; N: Non-tumor.

of mutation were 67aa (8/19, all were gga→ggt sense mutations, Figure 3A) and 127aa (6/19, four were att→act missense mutations, Figure 3B, 1 was atc→att sense mutations, and 1 was direct linked-point HBV -nt1899 leading to deletions of amino acid at the HBx COOH-terminal end).

In contrast, the frequencies of HBx deletion mutation in HCC were significantly higher than the corresponding non-cancerous liver tissues (16/19 *vs* 4/19, $P < 0.001$). Among them, eight exhibited full-length HBx deletions, the other 8 were deletions with size ranging from 4aa to 150aa. The deletion of HBx COOH-terminal end was also detected in 6 of the latter (Figure 4). The COOH-terminal deletion of HBx was exhibited in 14 HCC samples, which is a major feature of HBx identified in tumor tissues.

In addition, a specific integration of HBx at GA-rich region of 17p13 locus (repeat region 56047..56210/rpt_family = "GA-rich") was also found in 8 of 16 HCC (Figure 5, Table 1). All of these also exhibited full-length HBx deletions. And the specific integration of HBx was more frequent in HCC than in the corresponding non-cancerous liver tissues (8/16 *vs* 1/16, $P = 0.015$). Finally, the translocation occurring between chromosomes 17 and 14 was obtained from HCC tissues from the no. 2 patient. We considered that a mispriming of the HBV primer may have occurred, so we rechecked the primer sequences and found no significant similarities in chromosome 17 using BLAST. The HBx gene was amplified and direct sequencing was duplicated in all samples, and identical results were obtained. A mispriming of HBV primer in the chromosome 17 was eliminated.

Genomic integrated HBV DNA in HCC tissues

To investigate whether the detected HBV DNA was integrated into the genome of the HCC tissues, genomic DNA was extracted and restricted by EcoRI or Hind III prior to Southern blot analysis. Results revealed that the bands of variable sizes were hybridized with the 32P-labeled HBV, HBc, HBx and Pre-S probe. These include the three band patterns: approximately more than 3.2 kb of integrated pattern, replicated pattern of less than 3.2 kb and mixed pattern (integrated coexistence with replicated) (Figure 6A). In 44 of 45 pair samples, the hybridized bands of HBV full gene and fragments were obtained in 45.5%-56.8% tumors and 40.9%-52.3% non-

cancerous tissues, respectively (Table 2). Hybridized bands of HBx and HBc revealed deletion at least in some cases compared to HBV full-length gene probe. None of HBV full gene and fragments showed single replicated pattern in HCC, indicating that HBx integration had taken place in partial HCC tissues.

Deleted mutation of HBc and HBx in HCC

To further confirm that the hybridized bands contained deleted mutation HBx, PCR amplification was carried out using specific primers on DNA extracted from genomic DNA in 45 HCC and their corresponding non-cancerous liver tissues. The fragment sizes include two patterns: approximately 531bp of wild type and less than 531 bp of mutant type in HBc, and approximately 1207 bp of wild type and less than 1207 bp of mutant type in HBc + HBx, respectively (Figure 6B). As shown in Table 3, deleted mutation of HBx was more frequent in HCC than that of HBc. The amplified products were consistent with the genomic Southern blot analysis.

DISCUSSION

HBV infection is a major factor contributing to the development of HCC in China and the mutation in HBx plays an important role in this process^[2,19-22]. However, analysis of HBx sequence in tumor tissues of HCC patients from mainland China has not been done^[14,16,22,23]. A previous study of sera from 67 HCC patients from Taiwan indicates that 52% of samples contain HBx mutations^[23]. Our present study showed that the frequency of the HBx gene mutation in either tumor tissues or the corresponding non-cancerous liver tissue samples of HCC is very high, 57.9% and 94.7%, respectively. The most frequent spots of mutation identified are 67aa (8/20) and 127aa (6/20). The hot spots reported by other groups are nt. 382-389 (codons 128-130) in HCC samples collected from Qidong, China^[14], nt. 204 and 260-264 (codons 68 and 87-88) in HCC samples from Hong Kong^[16] and nt. 93 (codon 31) in HCC samples from Taiwan^[23]. These suggested that the HBx may have its own distinguished patterns of mutation in different geographic regions. As was reported by Chen GG *et al*, the biological consequence may be the same in all these regions^[16].

The most important finding of this study was the identification of HBx deletion mutation in our samples. HBx deletion was detected in 16 HCC samples, and 14 exhibited the COOH-terminal deletion of HBx in HCC tissues. A COOH-terminal deletion in the HBx gene was found in 5 of 9^[24] and 5/6 HCCs^[15]. There may be three regions of the X gene essential for the transactivation function of the X protein (at codons 46-52, 61-69 and 132-139)^[14,25]. All of these results suggest that HBx mutants with a COOH-terminal deletion were significantly correlated with the development and progression of HCC.

Although the mechanism involved in pathogenesis of HBx mutants with a COOH-terminal deletion remains largely unknown, some studies showed that HBx deleted mutants isolated from tumor tissues abrogated both

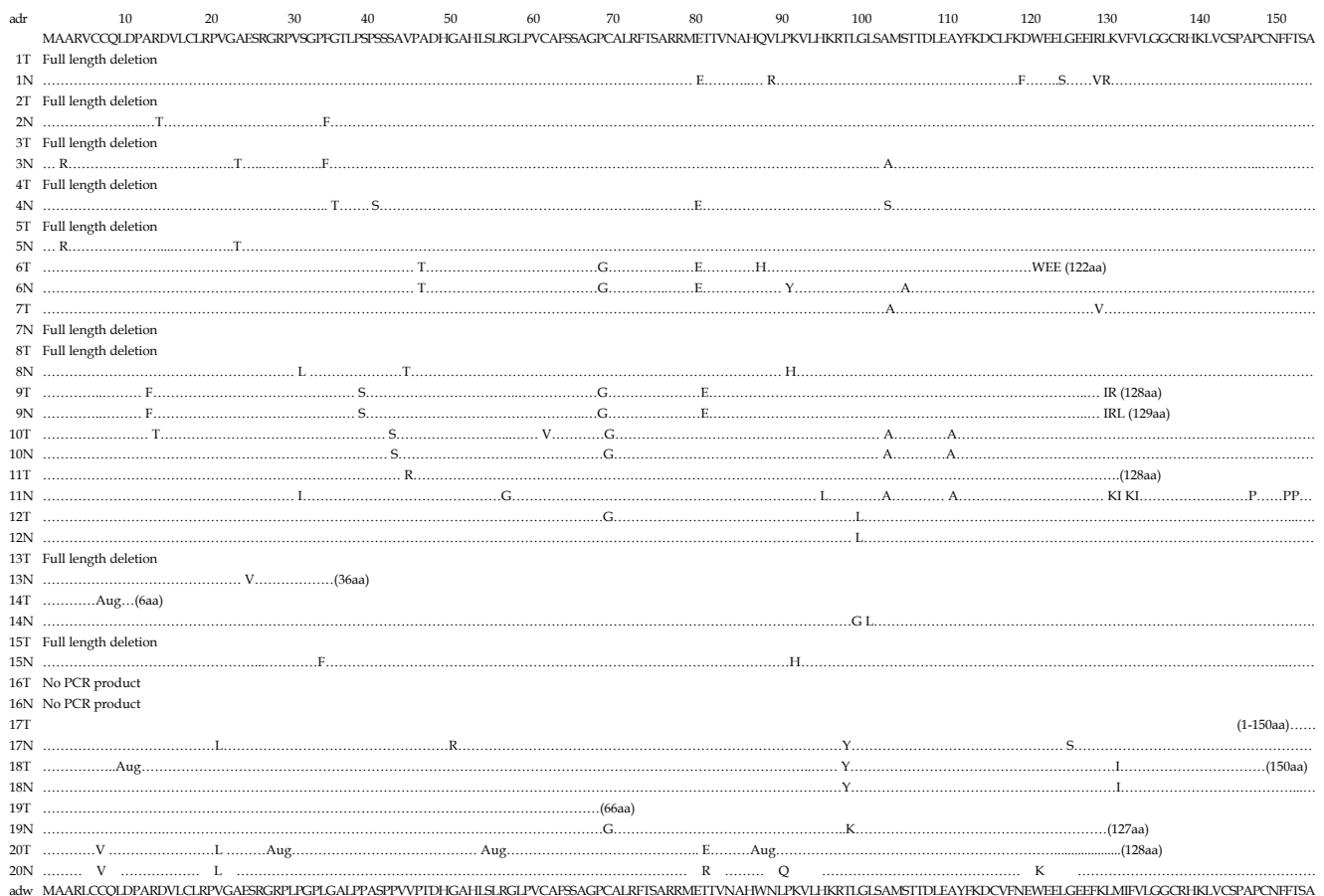


Figure 2 HBx sequencing in cancer and non-cancerous tissues from 20 HBV-associated HCC patients. The amino acid sequences of HBV adr and adw subtypes are shown at the top or the bottom. Identical amino acid residues are represented by dots. The underlined amino acids were deduced from cellular flanking sequences. The frequencies of HBx point mutations were significantly lower in HCC than in the corresponding non-cancerous liver tissues (11/19 vs 18/19, $P = 0.019$). T: Tumor; N: Non-tumor. "...": Represent the corresponding PCR amplified base sequences.

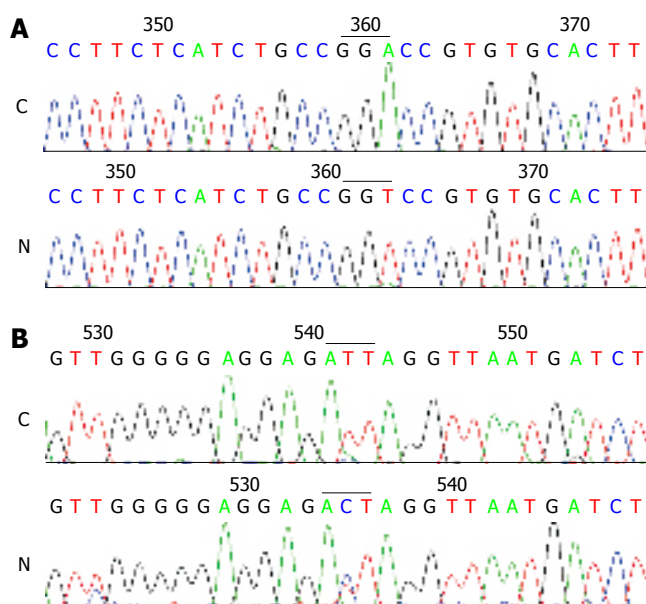


Figure 3 (A) GGA→GGT sense mutations at 67aa and (B) ATT→ACT mis-sense mutations at 127aa in non-cancerous liver tissues. C: Control; N: Non-tumor.

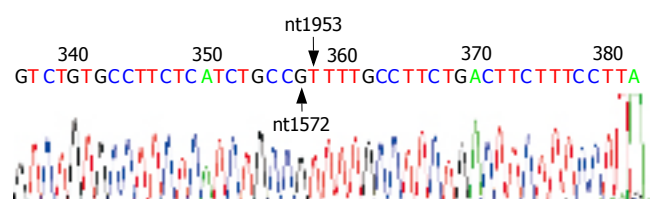


Figure 4 Sequencing of HBx deletion mutation in HCC.

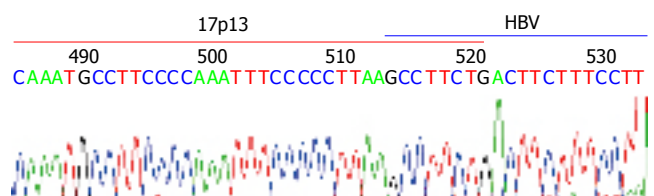


Figure 5 Sequencing of a specific integrated HBV at GA-rich region of 17p13 locus from HCC tissues of no. 2 patient. A: Tumor; B: Non-tumor.

Table 1 Sequences of HBx integrated with Homo sapiens chromosomal 17 nucleotide sequences in 8 HCC cases

Case No.	Homo sapiens chromosome 17, clone RP11-104H15 (PCR sequencing)			HBV gene
	A	B	C	
2	chromosome 14 (39-87)	56455-56249 (224-430)	56194-56158 (485-521)	1957-1976 (514-533)
3	56677-56472 (1-206)	56455-56249 (223-429)	56194-56158 (484-520)	1957-1976 (513-532)
4	56663-56472 (1-192)	56455-56249 (209-415)	56194-56158 (470-506)	1957-1976 (499-518)
5	56676-56472 (1-205)	56455-56249 (222-428)	56194-56158 (483-519)	1957-1976 (512-531)
8	56675-56472 (6-208)	56455-56249 (225-431)	56194-56158 (486-522)	1957-1976 (515-534)
13	56679-56472 (1-208)	56455-56249 (225-431)	56194-56158 (486-522)	1957-1976 (515-534)
15	56669-56472 (1-198)	56455-56249 (215-421)	56194-56158 (476-512)	1957-1976 (505-524)
16	56675-56472 (1-204)	56455-56249 (221-427)	56194-56158 (482-518)	1957-1976 (511-530)

A, B and C represent 3 discontinued sequences at 17p13 and integrated HBV gene sequences.

Table 2 Patterns of genomic integrated HBV DNA in HCC tissues by Southern blot

Gene probes	n	Tumor					Non-tumor liver tissues				
		I	R	I + R	Total	%	I	R	I + R	Total	%
HBV-DNA (EcoR I restriction)	44	16	0	9	25	56.8	10	5	8	23	52.3
HBV-DNA (Hind III restriction)	44	18	0	7	25	56.8	13	6	4	23	52.3
HBx-DNA (EcoR I restriction)	44	13	0	7	20	45.0	8	4	6	18	40.9
HBc-DNA (EcoR I restriction)	44	16	0	6	22	50.0	9	5	5	19	43.2
Pre-S (EcoR I restriction)	44	16	0	9	25	56.8	10	5	7	22	50.0

I: Integrated pattern; R: Replicated pattern, I + R: Integrated coexistence with replicated (mixed pattern).

the transactivation and antiproliferative effects of wild type HBx^[24]. When HBx deleted mutant plasmids were transfected to murine and human cell lines, a strongly increased colony formation, accelerated cell cycle progression, and synergetically promoted ras and myc transforming capacity were confirmed^[7,24,26]. Therefore, a COOH-terminal deletion may alter the balance of HBx functional domains in regulating cell proliferation and apoptosis, viability, and transformation. In addition, as previously reported^[15,27], we also noted the coexistence within the same tumor cells of full-length and COOH-terminally deleted HBx sequences, encoded by free or integrated HBV genome sequences. Although we do not exclude the coexistence within the same tumor cells of full-length and COOH-terminally deleted HBx sequences encoded by free or integrated HBV genome, we are investigating the biological implication of the COOH-terminally truncated HBx sequences.

Integration of HBV DNA was found in HCC at

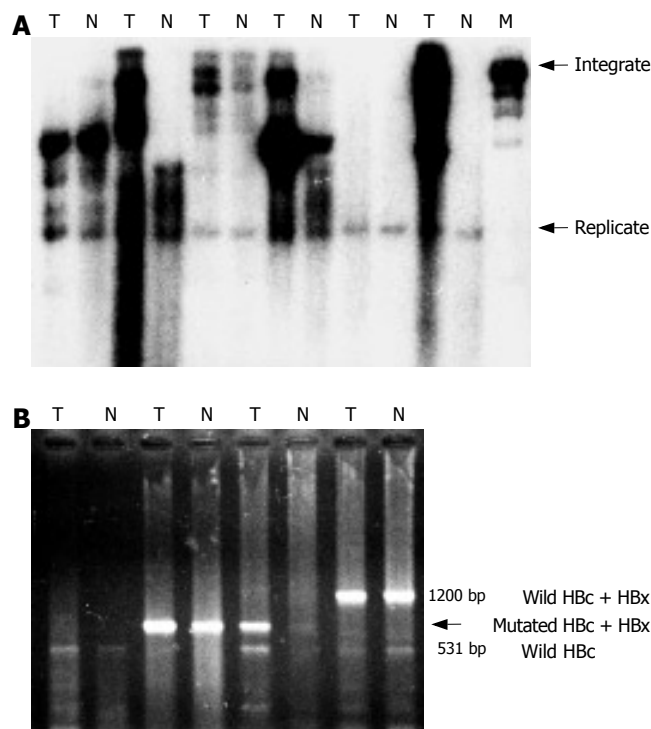


Figure 6 A: Detection of integrated HBx fragments in HCC tissues by Southern blot analysis; B: HBc and HBx fragment amplified by PCR. M: Marker; T: Tumor; N: Non-tumor.

GA-rich region of 17p13 locus in our study. We have eliminated the mispriming of HBV primer, and identified a specific integration of HBV DNA. This region includes the p53 gene, which is bound to and inactivated by HBxAg prior to tumor formation and then lost during tumor progression^[19]. This region also encodes microRNAs-22, -132, -195, -212^[19]; ubiquitin-conjugating enzyme E2G 1 and ubiquitin specific protease^[28]. We have also noted that most of the samples contained COOH-terminally deleted mutants of the HBx, and the specific integration of HBx was more frequent in HCC than in the corresponding non-cancerous liver tissues, and HBV integration including HBx, occurred in partial HCC tissues by Southern blot. These changes, to the best of our knowledge, have not been described elsewhere. Almost all of the HBV-associated HCCs harbor chromosomally integrated HBV DNA sequences, including chromosome 17p12-13^[4,19,29,30]. HBV integration can induce deletions in the host chromosome at the integration site^[19]. It was also recently confirmed that HBV insertion into cellular genes is a frequent event and that integration can occur in genes regulating cellular signal transduction cascades, proliferation control and cell viability^[7,19]. Thus, the putative HBV-specific integration sites, the biological impacts and the process with functional genomics of HBV associated HCCs need further studies.

In conclusion, HBx deletion, especially the COOH-terminal deletion of HBx, is a frequent event in HBV-associated HCC tissues. HBV integration including HBx, occurred in partial HCC tissues. This supports the hypothesis that the deletion and probably integrated forms of the HBx gene may be implicated in liver carcinogenesis.

Table 3 Deleted mutation of HBc and HBx in HCC by PCR, *n* (%)

Item	<i>n</i>	Tumor			Non-tumor		
		Positive	Wild type	Mutant type	Positive	Wild type	Mutant type
HBc	45	38 (84.4)	28 (73.7)	10 (26.3)	38 (84.4)	31 (81.6)	7 (18.4)
HBc + HBx	45	32 (71.1)	8 (25.0) ^b	24 (75.00) ^b	31 (68.9)	9 (29.0) ^b	22 (71.0) ^b

^b*P* < 0.01.

COMMENTS

Background

The hepatitis B virus (HBV) x protein (HBx) plays a critical role in the molecular pathogenesis of hepatocellular carcinoma (HCC). However, the mechanism remains largely unknown.

Research frontiers

HBx gene containing substantial but different deletions in the COOH-terminal region was found in many HBV-infected HCC patients from Qidong, Hong Kong and Taiwan, China.

Innovations and breakthroughs

The frequencies of HBx point mutations were significantly lower in HCC than their corresponding non-cancerous liver tissues. HBx deletion, especially the COOH-terminal deletion of HBx is a frequent event in HBV-associated HCC tissues in China. Integrated or integrated coexistence with replicated pattern were obtained in HCC and non-cancerous tissues.

Applications

Deletion and probably integrated forms of the HBx gene may be implicated in liver carcinogenesis.

Peer review

This study showed that HBx deletion, especially the COOH-terminal deletion of HBx is a frequent event in HBV-associated HCC tissues in China. This study confirmed their existence of HBx COOH-terminal deletion in human HCC, and provided some new information to clarify the mechanism in hepatocarcinogenesis.

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Expression of tumor necrosis factor-alpha converting enzyme in liver regeneration after partial hepatectomy

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Abstract

AIM: To study the expression of tumor necrosis factor-alpha converting enzyme (TACE) and evaluate its significance in liver regeneration after partial hepatectomy *in vivo*.

METHODS: Male SD rats underwent 70% partial hepatectomy. The remaining liver and spleen tissue samples were collected at indicated time points after hepatectomy. TACE expression was investigated by Western blotting, immunohistochemistry, and serial section immunostaining.

RESULTS: Expression of TACE in liver and spleen tissues after partial hepatectomy was a time-dependent alteration, reaching a maximal level between 24 and 48 h and remaining elevated for more than 168 h. TACE protein was localized to mononuclear cells (MNC), which infiltrated the liver from the spleen after hepatectomy. The kinetics of TACE expression was in accordance with the number of TACE-staining MNCs and synchronized with those of transforming growth factor- α (TGF α). In addition, TACE-staining MNC partially overlapped with CD3⁺ T lymphocytes.

CONCLUSION: TACE may be involved in liver regeneration by pathway mediated with TGF α -EGFR in the cell-cycle progressive phase *in vivo*. TACE production and effect by paracrine may be a pathway of involvement in liver regeneration for the activated CD3⁺ T lymphocytes.

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Key words: Tumor necrosis factor-alpha converting enzyme; Liver regeneration; Partial hepatectomy

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Lin XM, Liu YB, Zhou F, Wu YL, Chen L, Fang HQ. Expression of tumor necrosis factor-alpha converting enzyme in liver regeneration after partial hepatectomy. *World J Gastroenterol* 2008; 14(9): 1353-1357 Available from: URL: <http://www.wjg-net.com/1007-9327/14/1353.asp> DOI: <http://dx.doi.org/10.3748/wjg.14.1353>

INTRODUCTION

Tumor necrosis factor-alpha converting enzyme (TACE) is a kind of metalloprotease disintegrins, also known as ADAM17, which is a modular transmembrane protein with a zinc-dependent catalytic domain^[1]. TACE was originally cloned and named for its ability to cleave and convert tumor necrosis factor- α (TNF α) into a soluble form. Since then, TACE has been demonstrated to solubilize a variety of substrates including transforming growth factor- α (TGF α), members of the membrane-bound epidermal growth factor (EGF) family ligands, both TNFR- I and TNFR- II, and macrophage/colony-stimulating factor receptor^[2]. Liver regeneration after partial hepatectomy is very intricate. The process requires the activation of more than 100 genes and involves multiple cytokines and growth factors such as interleukin- I, hepatocyte growth factor (HGF), TNF α , TGF α , heparin-binding epidermal growth factor-like growth factor (HB-EGF) *etc*^[3,4]. It has been well documented that TACE mRNA and TACE protein are enhanced in several human malignant diseases such as breast cancer, lung cancer, and liver cancer^[5,6]. In a study of hepatocyte replication, Gretchen *et al* employed the AML-12 hepatocyte cell line and implicated the participation of TACE in hepatocyte replication by activating the TGF α -EGFR pathway^[7]. Hence, it is speculated that TACE is involved in cell proliferation and carcinogenesis in addition to inflammation^[8-10]. It was also reported that the activity of several metalloproteinases increased during liver regeneration^[11]. However, to date, there has been few studies on the correlation of TACE with liver regeneration after partial hepatectomy *in vivo*. To gain further insight into the involvement of TACE in liver regeneration, we investigated the expression of TACE in both liver and spleen tissues with a rodent partial hepatectomy model.

MATERIALS AND METHODS

Animals and study protocol

All animal experiments were performed following the

institution's criteria for the care and use of laboratory animals in Zhejiang University, China. Male SD rats 200-250 g) were fed standard rodent chow and water *ad libitum* in a temperature-controlled room. Rats were anesthetized with ether and underwent 70% partial hepatectomy according to the method of Higgins and Anderson. At indicated time points after hepatectomy, laparotomy was performed on the rats, and liver and spleen tissue samples were collected. A tissue sample was flash-frozen in liquid nitrogen for Western-blotting analysis, and the remainder was fixed in 4% formaldehyde and embedded in paraffin for immunohistochemical analysis. This study protocol was approved by the Ethics Committee of Zhejiang University, China.

Immunohistochemical analysis

Four μm -thick paraffin sections of liver and spleen tissue samples were cut. After deparaffinization, the endogenous peroxidase activity was blocked by placing the slides in methanol containing 3% (w/v) H_2O_2 for 30 min at room temperature. Normal goat serum was added and kept at room temperature for 15 min. The primary antibodies, rabbit anti-TACE polyclonal antibodies (1:100 dilution; CHEMICON) and mouse monoclonal anti-CD3 antibodies (1:200 dilution; Acris) were applied overnight at 4°C. After the slides were washed in phosphate buffered saline, the Envision + R system labelled polymer-HRP (Dako; Cytomation) was added and visualized using the DAB chromogen (Merck; Germany). Counterstaining of cell nuclei was accomplished with Mayer's hematoxylin (Sigma). Finally, sections were counterstained with hematoxylin, dehydrated, coverslipped, and evaluated microscopically. Positive-staining cells were counted for each visual field at 400 \times magnification.

Western blot analysis

For total protein extraction, samples of rat liver and spleen tissues were homogenized in NETN buffer supplemented with protease inhibitors and centrifuged at 15000 r/min for 60 min at 4°C. Homogenates containing 50 μg of protein were loaded. The proteins were size-separated by electrophoresis on 7.5% polyacrylamide gels (Bio-Rad), and then transferred onto PVDF membranes (Bio-Rad). After blocking, membranes were incubated with a rabbit polyclonal antibody against rat TACE (1:1000 dilution; CHEMICON), and then with an alkaline phosphatase-conjugated anti-rabbit antibody (1:5000 dilution; Amersham). Immunoreactive proteins were detected with a fluorescence scanner (Storm, Pharmacia) using ECF substrate according to the manufacturer's instructions (Amersham). In control experiments, the membrane was incubated with a mouse monoclonal anti- β -actin antibody (1:1000 dilution; Sigma) and with an alkaline phosphatase-conjugated anti-mouse antibody (1:5000 dilution; Amersham).

RESULTS

TACE expression localization and pattern in liver and spleen following partial hepatectomy

To investigate TACE protein localization and pattern, we examined liver tissue paraffin sections by immunohisto-

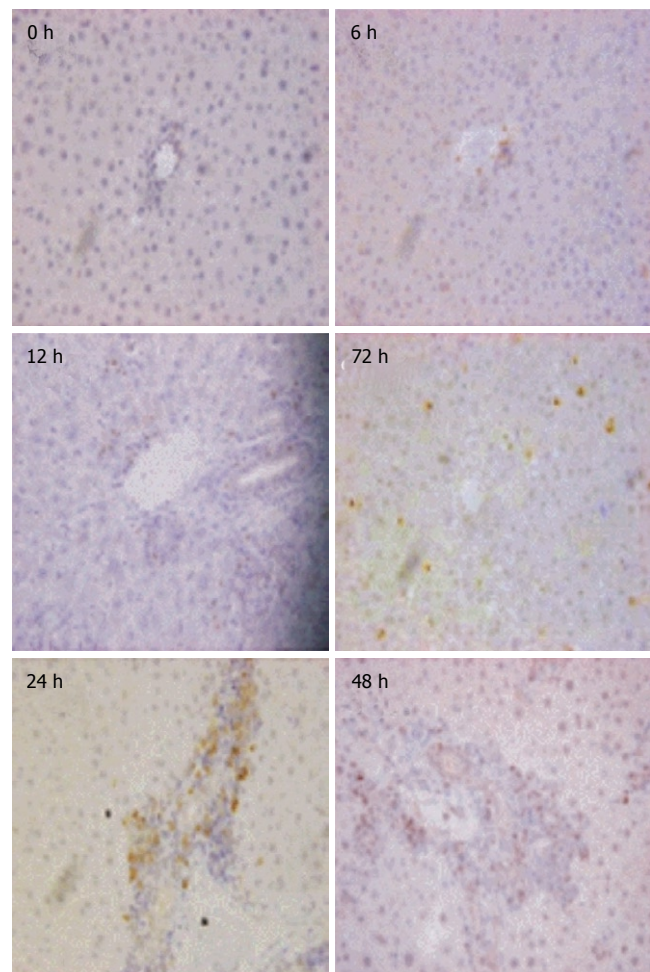


Figure 1 Rat liver stained using anti-TACE antibodies at various time points after hepatectomy. None of the parenchymal cells was stained at any of the time points. MNC accumulated markedly at periportal sites from 24 to 48 h after hepatectomy while intense TACE staining was seen. MNC declined and distributed to the intermediate regions after 72 h ($\times 400$).

chemistry. Mononuclear cells (MNC) stained positively, but hepatocytes, biliary epithelia cells, and endothelial cells did not. There were few MNC in portal triads, and TACE staining could be hardly detected prior to hepatectomy; however, after hepatectomy, the TACE-staining MNC was observed to infiltrate to periportal sites. Marked accumulation of MNC was found at periportal sites from 24 to 48 h, while intense TACE-staining MNC was visible. The TACE-staining MNC became less abundant and distributed to the intermediate regions after 72 h (Figure 1). Because many TACE-staining MNCs were detected in the portal vein and it was speculated that the MNC mainly came from the spleen through portal vein current (Figure 2), we performed further immunohistochemical studies on spleen tissues. The kinetic feature of TACE-staining in spleen is analogous to that in liver, while the TACE-staining MNC reached the marginal zone and splenic sinus (Figure 3). The quantities of TACE-staining MNC at various time points in liver and spleen of 7 rats in each group were counted randomly under microscopy (Figure 4).

TACE expression level in liver and spleen following partial hepatectomy

The TACE expression levels in liver and spleen tissues at

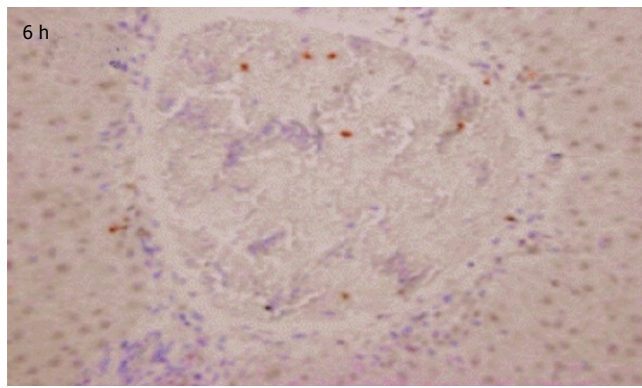


Figure 2 The TACE stained MNC in portal vein current 6 h after hepatectomy. The MNC infiltrated to periportal sites through portal triad vein endothelial cells ($\times 400$).

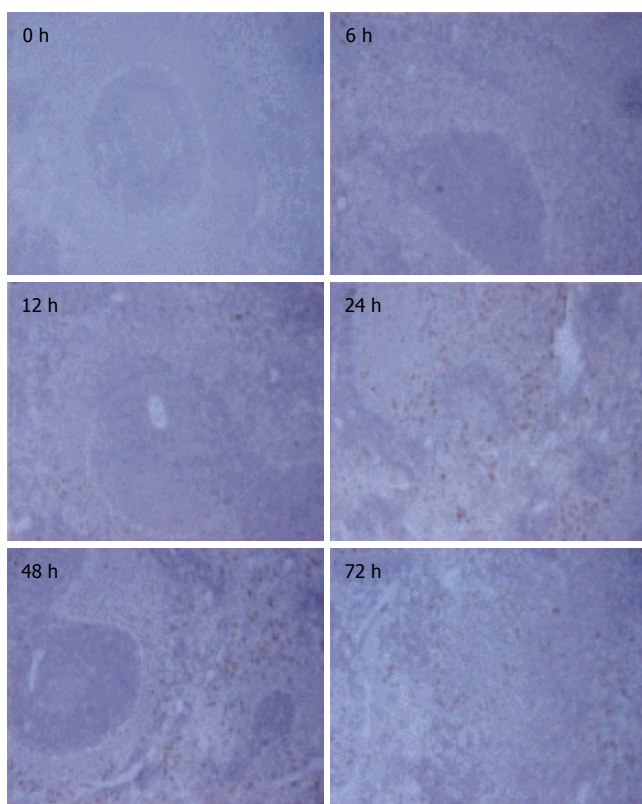


Figure 3 Immunostaining of TACE in rat spleen tissue at various time points. The TACE-stained MNC increased, reaching a peak at similar time points with liver. The positively stained MNC distributed at the marginal zone and splenic sinus. Numbers indicate the time points after hepatectomy ($\times 400$).

various time points were evaluated by Western blotting. Because the antibody was directed against the cytoplasmic domain of the protein, both the precursor (pro-TACE) and the mature forms were detected. As shown in Figure 5, TACE expression in the liver and spleen after hepatectomy is a time-dependent alteration, reaching a maximal level between 24 h and 48 h. The TACE level declined from 72 h, but remained elevated for more than 168 h as compared with the pre-hepatectomy level in liver tissues.

Correlation of TACE-staining MNC with T lymphocytes

To identify the stained MNC, we immunostained serial paraffin sections of liver tissues at 48 h after hepatectomy

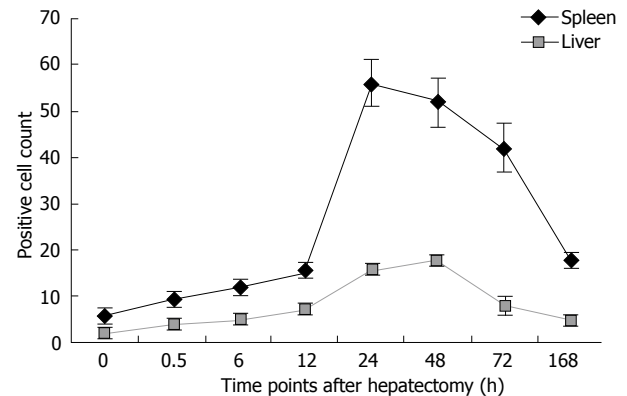


Figure 4 TACE-positive MNC in liver and spleen at various time points after hepatectomy. Data are mean \pm SD, $n = 7$ animals in each group.

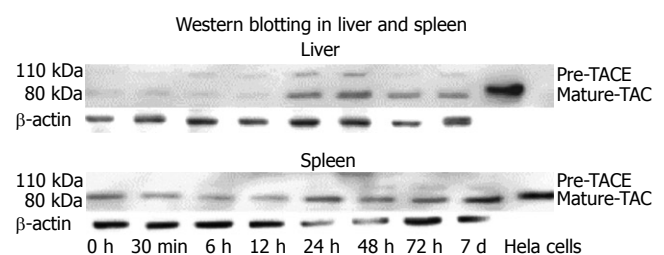


Figure 5 TACE protein level in rat liver and spleen following partial hepatectomy. Rat liver and spleen tissue samples taken at various time points were processed and subjected to Western blot analysis using anti-TACE antibodies. The heLa cells were lysed and the proteins were loaded as a positive control. Expression of TACE in liver and spleen after hepatectomy is a time-dependent alteration, reaching a maximal level between 24 and 48 h.

using an anti-CD3 antibody. This experiment was done in light of the report that during liver regeneration, extrathymic CD3⁺ T cells in liver are significantly activated in terms of increases in both proportion and absolute number. The feature demonstrated that the CD3⁺ T lymphocytes partially overlapped TACE staining MNC (Figure 6).

DISCUSSION

Hepatocyte replication comprises two phases: priming and cell-cycle progression^[12]. TNF α and interleukin-6 are the main cytokines triggering hepatocyte progression from G0 to G1. HB-EGF and TGF α play an important role in cell-cycle progression. Both TGF α and HB-EGF are ligands of the EGF family and are primary mitogens for hepatocyte proliferation in culture^[13,14]. It is believed that the functions of TGF α and HB-EGF at least partially overlap during liver regeneration^[15,16]. Enhanced expression of TGF α mRNA in hepatocytes peaks in 24 h and remains elevated for at least 48 h after hepatectomy^[17]. TGF α anchored to the cell membrane in precursor form is cleaved by TACE and then binds to EGFR, which activates a phosphorylation cascade leading to DNA replication. The mitogenic cascade involves ERK1/2 and PKB. TNF α enlarges TACE activation by shedding the precursor of TGF α ^[18]. TGF α is produced by hepatocytes and functions through an autocrine mechanism. TGF α and EGF play a

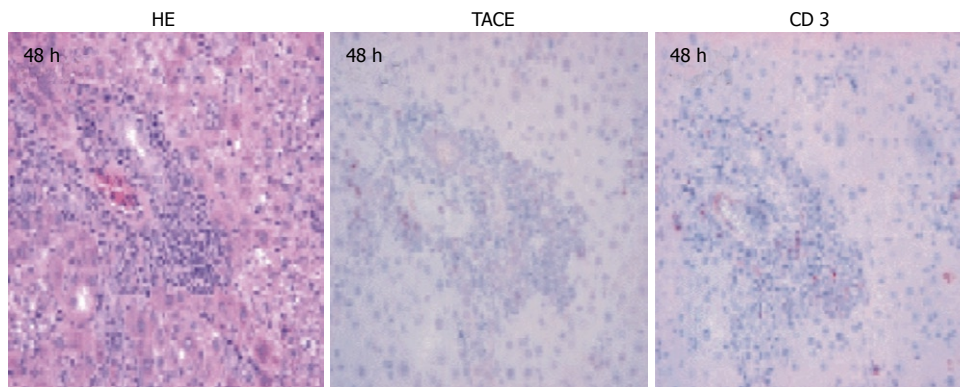


Figure 6 The serial paraffin sections of liver tissue at 48 h after hepatectomy were stained with HE and immunostained with anti-TACE antibodies and anti-CD3 antibodies. TACE-positive MNC partially overlapped with CD3⁺ T lymphocytes ($\times 400$).

major role in the progressive phases of liver regeneration after hepatectomy^[19-21].

TACE is mediated by furin and related proprotein convertases. TACE was originally cloned and named for its ability to cleave and convert TNF α into a soluble form. Cells such as macrophages, lymphocytes, and monocytes, which all produce abundant TNF α , are believed to express TACE enzyme^[22]. TACE is found to be involved in carcinogenesis by TGF α , and the HB-EGF-EGFR pathway. Distinct ADAM metalloproteinases regulate G protein-coupled receptor-induced cell proliferation and survival^[23,24]. The TACE inhibitor TAPI-1 interferes with TGF α release into the culture medium and subsequent EGFR signalling through ERK1/2 and PKB, thereby blocking DNA replication^[25]. These data substantiate the idea that TACE plays a significant role and forms a link between cytokine and growth factor pathways in cell proliferation.

In the present study, we examined the kinetic level of TACE expression and localization following partial hepatectomy *in vivo*. It demonstrated that the kinetics of TACE were relatively well synchronized with those of TGF α and hepatocyte proliferation^[3,15,26]. After hepatectomy in rats, the first peak of DNA synthesis in hepatocytes occurs at about 24 h, with a smaller peak between 36 h and 48 h. The other cells of the liver enter into DNA synthesis at 48 h or later^[12]. Although TACE is essential for cleaving TNF α and over-expression of TACE promotes inflammation by producing excessive soluble TNF α , our study shows that the kinetics of TACE expression are not compatible with those of TNF α in liver regeneration. TNF α increases abruptly and reaches a peak in the priming phase^[12,27], but TACE rises to a peak from 24 h to 48 h post-hepatectomy. Such scenarios may suggest that TACE is involved in liver regeneration by pathways including TGF α -EGFR in the cell-cycle progressive phase, but not by the TNF α pathway. It was also reported that during liver regeneration, extrathymic CD3⁺ T cells in the liver are significantly activated in terms of both increases in proportion and absolute number. This activation was observed at an early phase (d 2) of liver regeneration^[28,29]. IL-1 and TNF α , which are produced by activated kupffer cells and sinusoidal endothelial cells, can induce the activation of T-cell differentiation. The mechanism of T-cell activation of hepatocyte proliferation is unequivocal^[30]. In this research, immunohistochemical study using serial sections showed TACE-staining

MNC partially overlaps with CD3⁺ T lymphocytes. It is conceivable that TACE production and effect by paracrine may be a pathway of involvement in liver regeneration for activated CD3⁺ T lymphocytes.

COMMENTS

Background

Tumor necrosis factor-alpha converting enzyme (TACE) is a kind of metalloprotease disintegrins that acts to solubilize a variety of substrates including tumor necrosis factor- α (TNF α), transforming growth factor- α (TGF α), epidermal growth factor (EGF) family and has been considered to involve in carcinogenesis by TGF α , heparin-binding epidermal growth factor-like growth factor (HB-EGF) pathway. It was also reported that the activity of several metalloproteinases increase during liver regeneration.

Research frontiers

Liver regeneration after partial hepatectomy is very intricate. It involves expression of multiple cytokines and growth factors such as HGF, TNF α , TGF α and HB-EGF.

Innovations and breakthroughs

To date, there has been few studies on the correlation of TACE with liver regeneration after partial hepatectomy *in vivo*. To study the expression of TACE during liver regeneration, we investigated the liver and spleen tissues by a rodent model with partial hepatectomy. It demonstrated that TACE was produced by the activated CD3⁺ T lymphocytes and the kinetic expression of TACE was well synchronized with that of TGF α and hepatocyte proliferation.

Applications

TACE is implicated in liver regeneration by the TGF α pathway that overlaps partially with carcinogenesis.

Peer review

The authors investigated the expression of TACE during liver regeneration in rats after 70% partial hepatectomy. They observed a time dependant expression with a peak between 24 h and 48 h. They located TACE to mononuclear cells. The authors conclude that TACE expression is synchronized with TNF-alpha and that TACE is expressed on mononuclear cells.

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CLINICAL RESEARCH

Prevalence of gastroesophageal reflux symptoms in a large unselected general population in Japan

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Abstract

AIM: To examine the prevalence of gastroesophageal reflux disease (GERD) symptoms in a large unselected general population in Japan.

METHODS: In Japan, mature adults are offered regular check-ups for the prevention of gastric cancer. A notice was sent by mail to all inhabitants aged > 40 years. A total of 160983 Japanese (60774 male, 100209 female; mean age 61.9 years) who underwent a stomach check up were enrolled in this study. In addition, from these 160983 subjects, we randomly selected a total of 82894 (34275 male, 48619 female; mean age 62.4 years) to evaluate the prevalence of abdominal pain. The respective subjects were prospectively asked to complete questionnaires concerning the symptoms of heartburn, dysphagia, and abdominal pain for a 1 mo period.

RESULTS: The respective prevalences of the symptoms in males and females were: heartburn, 15.8% vs 20.7%; dysphagia, 5.4% vs 7.8%; and abdominal pain, 6.6% vs 9.6%. Among these symptoms, heartburn was significantly high compared with the other symptoms, and the prevalence of heartburn was significantly more frequent in females than in males in the 60-89-year age

group. Dysphagia was also significantly more frequent in female patients.

CONCLUSION: The prevalence of typical GERD symptoms (heartburn) was high, at about 20% of the Japan population, and the frequency was especially high in females in the 60-89 year age group.

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Key words: Gastroesophageal reflux disease; Heartburn; Dysphagia; Abdominal pain

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INTRODUCTION

Heartburn and acid regurgitation, typical symptoms of gastroesophageal reflux disease (GERD), are considered to be reasonably specific for diagnosis^[1,2]. The dominant mechanism of GERD is contact of the esophageal mucosa with acid, and GERD can also produce symptoms or signs of tissue injury within the oropharynx, larynx and respiratory tract. Recent studies have suggested GERD causes a wide spectrum of non-esophageal conditions including asthma, non-obstructive dysphagia, and non-cardiac chest pain^[3,4]. Moreover, it has been reported that acid-suppressive therapy resolves heartburn and epigastric pain in GERD patients^[5,6]. Therefore, epigastric pain may be a clinical symptom of GERD.

GERD is a common condition and its prevalence

varies in different parts of the world. Several population-based studies have reported its prevalence is more frequent in Western^[7-10] than in Eastern countries^[11,12]. According to the reports from the Ministry of Health and Welfare in 1994, the incidence of heartburn in Japan was only about 2% of the population who complained of symptoms. A recent study from Japan has reported patients complaining of heartburn account for 24.7%-42.2%^[13-17]. All of these previous studies were performed on subjects who visited the hospital as outpatients or for routine physical examination^[13-17]. However, we have to consider the possibility that outpatients are symptomatic, rather than a true random sample of the population. Furthermore, subjects undergoing their annual medical check-up make a periodic visit to each of the medical institutions for routine physical examination. These subjects may have caused some bias, and subjects who visited the hospital as outpatients or for routine physical examination may not be similar to the general population. Therefore, we decided to collect data from subjects attending a primary health care institution that was conducting an annual medical check-up, rather than using data from hospital-based subjects.

In Japan, mature adults are offered regular check-ups for the prevention of gastric cancer. A notice was sent by mail to all inhabitants aged > 40 years. Thus, the majority of subjects in this study were selected according to their age, and we considered this study sample might be more similar to the general population than those of previous studies, and in this respect, this study is considered to be the first to highlight the prevalence of GERD symptoms in a large unselected Japanese population. This is believed to be the first study to target > 100 000 unselected subjects in Japan, with the aim of: (1) examining the prevalence of heartburn as a typical symptom of GERD and dysphagia as an atypical symptom of GERD; and (2) identifying an association between GERD symptoms and abdominal pain.

MATERIALS AND METHODS

Health check up in Miyagi prefecture, Japan

The population of Miyagi prefecture comprises 1 889 683 adults (909 795 male, 979 888 female), and there are 1 236 724 adults aged > 40 years old (579 822 male, 656 902 female). In Japan, mature adults are offered regular check-ups for the prevention of gastric cancer. Especially in Miyagi prefecture, those over the age of 40 years are recommended to undergo regular health check-ups. A notice was sent by mail in either the first or second half of the year to all inhabitants aged > 40 years who lived in the prefecture. A list of medical institutions was also included so that the subjects could choose where to have the general check-up. Even those subjects under 40 years old could take the check-up if they so desired.

Study subjects

The center chosen for this study was the Institute of the Miyagi Cancer Society. A total of 160 983 subjects (60 774 male, 100 209 female, mean age 61.9 ± 11.1 years) who underwent a regular check-ups for the prevention of gastric cancer at the above center between January and December

Table 1 Questionnaire used in this study

Please circle the appropriate responses for each of the following symptoms during a 1-mo period

- | | | |
|---|---|-------|
| (1) Do you suffer from the symptom of dysphagia? | 1: yes | 2: no |
| | (a: usually; b: sometimes) | |
| | (c: throat; d: chest; e: stomach) | |
| (2) Do you suffer from the symptom of heartburn? | 1: yes | 2: no |
| | (a: usually; b: sometimes) | |
| (3) Do you suffer from the symptom of abdominal pain? | 1: yes | 2: no |
| | (a: usually; b: sometimes) | |
| | (f: before eating; g: after eating; h: no relation) | |

2003 were enrolled in this study in a prospective fashion. The 160 983 subjects were divided into seven geographical areas, according to the place of residence of the inhabitants, namely Sendai, Tome, Sennan, Kesennuma, Isinomaki, Osaki, and Kurihara. Among these seven geographical areas and 160 193 subjects, we additionally randomly selected a total of 82 894 subjects (34 275 male, 48 619 female, mean age 62.4 ± 11.0 years) to evaluate the prevalence of abdominal pain. Subjects were assigned to the following age groups: 30-39, 40-49, 50-59, 60-69, 70-79, and 80-89 years.

Methods questionnaire

Table 1 shows the questionnaire used in this study. The respective subjects were prospectively asked to complete the questionnaires concerning the symptoms of heartburn, dysphagia, and abdominal pain within a 1 mo period. The subjects were requested to simply answer "yes" or "no" to the symptoms, and if they answered "yes", they were further questioned on the frequency of symptoms ("usually" or "sometimes"). In addition, concerning the symptom of dysphagia, subjects were asked where it occurred (throat, chest or stomach). The relationship of abdominal pain with meals, if any, was elicited. This questionnaire was designed as a self-reporting instrument to measure symptoms experienced over the month previous to returning the completed questionnaire.

X-ray examination

In order to check for gastric cancer, X-ray examinations were also performed on all the enrolled subjects. In addition, we evaluated disease classification based on X-ray examination independently by two experienced gastrointestinal doctors. This was graded in the present study as: normal, gastric ulcer scar, duodenal ulcer scar, gastric and duodenal ulcer scar, hiatus hernia, and patients in whom further endoscopic testing was necessary, because they were suspected of having gastric cancer (cancer suspected). Hiatus hernia in this study was defined as a circular extension of the gastric mucosa of 3 cm or more above the diaphragmatic hernia^[18].

Statistical analysis

The 160 983 eligible individuals interviewed were considered as a representative sample of the Japanese population. The

Table 2 Disease classification by X ray examinations

Disease classification by X-rays	n (%)	Male/female
Normal	139167 (86.4)	(49338/89829)
Gastric ulcer scar (GU)	2222 (1.4)	(1648/574)
Duodenal ulcer scar (DU)	2346 (1.5)	(1192/1154)
Gastric & Duodenal scar (GDU)	434 (0.3)	(341/93)
Hiatus hernia (HH)	1198 (0.7)	(404/794)
Cancer suspected	15616 (9.7)	(8080/7536)
Total	160983 (100.0)	(60774/100209)

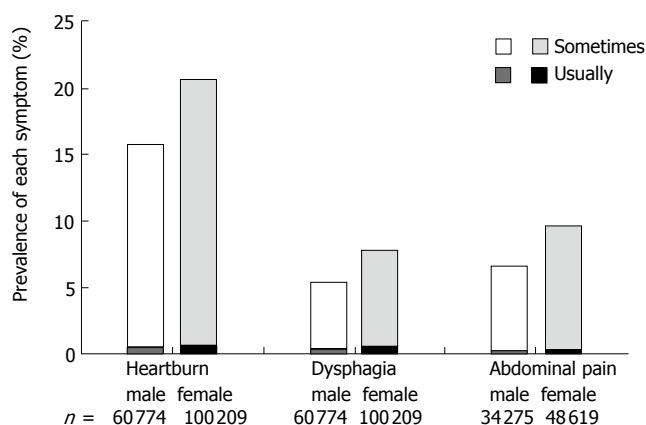


Figure 1 Prevalence of each symptom. Overall gender-specific prevalence rate for heartburn, dysphagia, and abdominal pain. Among these symptoms, heartburn was significantly high compared with the other symptoms. All symptoms were significantly more common in females than in males (χ^2 test, $P < 0.0001$ vs the symptom group).

results are given with a confidence interval of 95%. The values are expressed as frequency, and categorical variables were compared using the χ^2 test. $P < 0.05$ was considered significant.

RESULTS

The mean age of the sample population was 61.9 ± 11.1 years. About 62% of the interviewed subjects (100209) were women. A total of 160848 of the 160983 eligible subjects (99%) responded to the questionnaire on heartburn and dysphagia. Of the total of 82894 eligible subjects additionally randomly selected to evaluate the prevalence of abdominal pain, 82484 (99%) responded to this question.

The disease classification by X-ray examination used in this study and the numbers of subjects are shown in Table 2. Normal classification accounted for 86.4%, and the others in who further endoscopic testing was required because they were suspected of having gastric cancer accounted for 9.7%.

Frequency of respective symptoms

The overall and gender-specific prevalence rates for heartburn, dysphagia and abdominal pain are shown in Figure 1. The prevalence of the symptoms respectively in males and females were: heartburn, 15.8% (sometimes 15.3%, usually 0.5%) vs 20.7% (sometimes 20.1%, usually 0.6%); dysphagia, 5.4% (sometimes 5.0%, usually 0.4%) vs 7.8% (sometimes 7.3%, usually 0.5%); and abdominal pain,

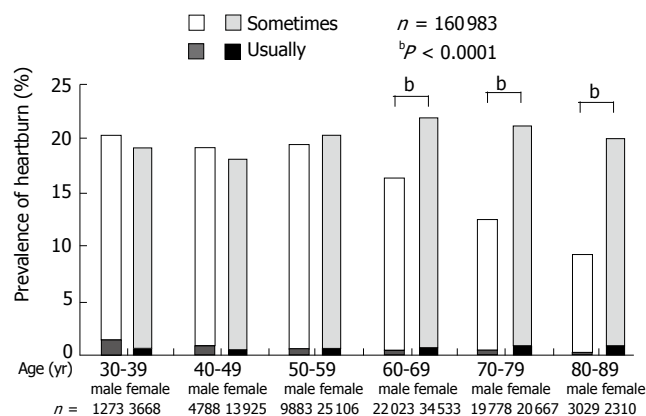


Figure 2 Age- and gender-specific prevalence rate for heartburn over a 1-month period. Heartburn was significantly high in females in the 60-89-year age group (χ^2 test, $P < 0.0001$ vs each generation group).

6.6% (sometimes 6.5%, usually 0.1%) vs 9.6% (sometimes 9.4%, usually 0.2%). Among these symptoms, heartburn was significantly high compared with the other symptoms, and all symptoms were significantly more common in females than in males.

Age- and gender-specific prevalence of respective symptoms

The age- and gender-specific prevalence of heartburn experienced in the month prior to completion of the questionnaires is shown in Figure 2. The prevalence of heartburn remained high with age among women, whereas among men it peaked around the 30-39 years age group, and thereafter declined. The prevalence of heartburn showed a significant age- and gender-related difference in patients in the 60-89 years age group, and the prevalence of heartburn was significantly more frequent in females than in males. The smaller subset of patients reporting "usually" for this symptom is also shown in Figure 2, which was also significantly more frequent in females than in males in the 60-89 years age group.

The age- and gender-specific prevalence of dysphagia experienced in the month prior to completion of the questionnaires is shown in Figure 3. Dysphagia was significantly more frequent in females than in males in all age groups.

The age- and gender-specific prevalence of abdominal pain experienced in the month prior to completion of the questionnaires is shown in Figure 4. Women were more symptomatic than males in all age groups. Abdominal pain was more prevalent in the younger 30-49 years age group than in the older age groups.

Association between typical (heartburn) and atypical (dysphagia) symptoms of GERD

Table 3 shows the prevalence of heartburn among subjects in the dysphagia "usually", "sometimes" and no dysphagia groups. The subjects with dysphagia had a significantly higher frequency of heartburn than those without it. Table 4 shows the prevalence of dysphagia among subjects with heartburn "usually", heartburn "sometimes", and no

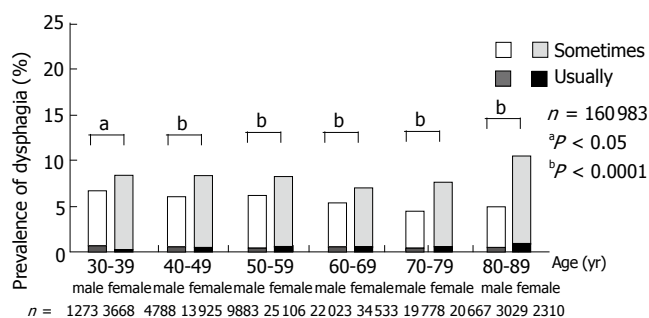


Figure 3 Age- and gender-specific prevalence rate for dysphagia over a 1 month period. Dysphagia was significantly more frequent in females than in males in all age groups (χ^2 test, $^bP < 0.0001$ and $^aP < 0.05$ vs each generation group).

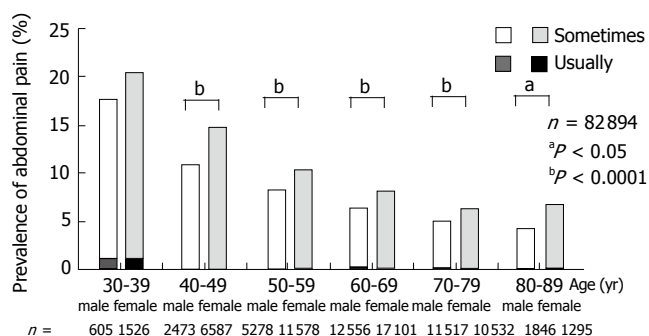


Figure 4 Age- and gender-specific prevalence rate for abdominal pain over a 1 month period. Women were significantly more symptomatic than men in all age groups, except 30-39 years (χ^2 test, $^bP < 0.0001$ and $^aP < 0.05$ vs each generation group).

heartburn. The subjects with heartburn had a significantly higher frequency of dysphagia than those without.

Prevalence of heartburn and dysphagia in relation to the frequency of abdominal pain

Table 5 shows the prevalence of heartburn and dysphagia among subjects in the abdominal pain “usually”, “sometimes” and no abdominal pain groups. The subjects with abdominal pain had a significantly higher frequency of heartburn and dysphagia than those without.

Correlation with disease classification

The prevalence of heartburn and dysphagia was significantly higher in both males and females with hiatus hernia than those without. The prevalence of abdominal pain was significantly higher in both males and females with gastric ulcer, duodenal ulcer, and gastric and duodenal ulcer scarring than in those who were scar-free.

DISCUSSION

This is believed to be the first study that has highlighted the prevalence of GERD symptoms in > 100 000 unselected subjects in a prospective fashion in Japan.

Heartburn has been reported to be specific for the diagnosis of GERD^[1,2]; therefore, we examined heartburn as a typical symptom of GERD. The most important finding was that the monthly prevalence of heartburn was significantly higher (20%) than that for dysphagia and

Table 3 The prevalence of heartburn in relation to the frequency of dysphagia

	Dysphagia (usually)	Dysphagia (sometimes)	No dysphagia
<i>n</i> = (male/female)	(247/563)	(2806/6875)	(57 476/92 298)
Heartburn (%)			
Male	43.7 ^b	48.7 ^b	13.9
Female	53.8 ^b	52.8 ^b	18.0

Chi-square test, $^bP < 0.001$ vs each “no dysphagia” group.

Table 4 The prevalence of dysphagia in relation to the frequency of heartburn

	Heartburn (usually)	Heartburn (sometimes)	No heartburn
<i>n</i> = (Male/female)	(327/680)	(8769/18 984)	(51 092/79 298)
Dysphagia (%)			
Male	33.3 ^b	15.7 ^b	3.3
Female	39.1 ^b	19.5 ^b	4.6

Chi-square test, $^bP < 0.001$ vs each “no heartburn” group.

Table 5 The prevalence of heartburn and dysphagia in relation to the frequency of abdominal pain

	Abdominal pain (usually)	Abdominal pain (sometimes)	No abdominal pain
<i>n</i> = (male/female)	(76/98)	(2344/4409)	(31 625/43 832)
Heartburn (%)			
Male	47.3 ^b	44.1 ^b	13.8
Female	53.0 ^b	48.0 ^b	17.7
Dysphagia (%)			
Male	28.9 ^b	21.1 ^b	4.1
Female	26.5 ^b	23.2 ^b	5.6

Chi-square test, $^bP < 0.001$ vs each “no abdominal pain” group.

abdominal pain in our Japanese general population.

GERD symptoms are common and its prevalence varies in different parts of the world. The study by El-Serag *et al*^[10] in 2004 used a questionnaire to survey 496 medical center staff for GERD symptoms. They reported that monthly prevalence of heartburn was 34.2%-40.6% in the United States. On the other hand, in Asia the prevalence has varied, but is generally lower than that in Western studies. Wong *et al*^[11] have reported that the prevalence of heartburn among 902 randomly selected people occurring at least monthly was 8.9%, while Cho *et al*^[12] have found a lower prevalence of 4.7% among 2209 randomly selected individuals.

In Japan, Mishima *et al*^[13] have designed a study to clarify the characteristics of GERD, based on 2760 subjects undergoing their annual medical check-up for gastric cancer. They reported that GERD was present in 17.9% of the Japanese people who presented for annual medical health checks. Fujiwara *et al*^[14] have reported the prevalence of heartburn among 6035 routine physical examinations was 24.7%. Watanabe *et al*^[16] have reported the prevalence of GERD among 4139 hospital outpatients was 37.6%. Another study in Japan by Ohara *et al*^[17] has

shown a total of 42.2% of Japanese individuals who visited hospital for routine physical examination and outpatients experienced heartburn. All of these previously mentioned studies were performed on subjects who visited a hospital as outpatients or for routine physical examination^[13-17]. However, we consider the possibility that outpatients are symptomatic, rather than a true random sample of the population. Furthermore, subjects who undergo their annual medical check-up make a periodic visit to each of the medical institutions for routine physical examinations. These subjects may have caused some bias, and subjects who visited the hospital as outpatients or for routine physical examinations may not be similar to the general population. On the other hand, in this study, the majority of the sample survey was selected according to age. As stated before, those over the age of 40 years are recommended to undergo regular health check-ups, and a notice was sent by mail to all inhabitants who live in Miyagi prefecture, according to their age. We considered this study sample might be more similar to the general population than that in previous studies^[13-17], and in this respect, this study is considered to be the first to highlight the prevalence of GERD symptoms in a large unselected Japanese population.

It is difficult to compare previous reports in terms of difference in the prevalence of GERD symptoms in Japan because of the diverse methodologies used. However, several studies have shown the prevalence of GERD symptoms has recently been gradually increasing^[15-17,19]. The prevalence of heartburn in this study was about 20%, which is considered to be higher than that of previous studies^[19].

There are various factors that might be responsible for the recent upward trend in the prevalence of GERD symptoms in Japan. Risk factors for GERD have been shown to include dietary intake^[20,21], smoking^[22], alcohol consumption^[23], high body mass index^[24], family history of reflux symptom^[25], aging population, and the presence of a hiatus hernia^[15,17]. For the first point, the average fat intake in Japan has increased over the past 20 years^[26]. Dietary fat has been shown to increase the frequency of transient lower esophageal sphincter relaxation^[21]. Secondly, the population has become increasingly overweight^[27], and obesity may lead to an increase in intragastric pressure^[15]. Thirdly, it has been reported Japanese patients have a high incidence of *H. pylori* infection and this leads to an atrophic pattern of gastritis with low acid secretion^[28,29]. The declining prevalence of *H. pylori* infection^[30], due to improved hygiene conditions and widespread use of eradication therapy in Japan could also have paradoxically contributed to the increased frequency of GERD. These may be reasons why the prevalence of GERD has recently increased in Japan.

GERD is also associated with a range of other atypical symptom originating in the esophagus, chest and respiratory tract. Several studies have reported approximately one-third of GERD patients suffer from these symptoms, such as dysphagia and chest pain^[3,4]. We also found considerable overlap among heartburn and dysphagia in this survey, and this suggests an association between GERD and dysphagia.

The age and gender relationships with GERD symptoms

are still controversial. A study in Japan by Ohara *et al*^[17] has shown GERD symptoms are more common among women, and are unrelated to age. In this study, typical and atypical symptoms were found to be common especially in women. Also, there was a strong relationship between age and gender and the prevalence of symptoms. Our results showed the prevalence of GERD symptoms remained high with age among women, whereas among men, it peaked around the 30-39 years age group, and declined thereafter. One possible reason for high prevalence of GERD in elderly women in Japan may be osteoporosis and kyphosis. Elderly Japanese women tend to have a higher incidence of both osteoporosis and kyphosis than in men the same age^[31]. Lumbar kyphosis is the major cause of stooped posture, and this may exacerbate the development of hiatus hernia, which is reported as a risk factor for GERD.

Some studies have reported acid suppressive therapy resolves epigastric pain in patients with GERD^[5,6]. According to these studies, epigastric pain might be one of the clinical symptoms of GERD. In this study, the symptoms of heartburn, dysphagia and abdominal pain also overlapped with each other, similar to previous studies.

The present study has several limitations. First, the main limitation is the fact that the questionnaire was not validated. We estimated the symptom frequency as "usually" or "sometimes". This may be considered vague terminology for precise evaluation of symptom prevalence. Individual subjects might differently interpret the terms of "usually" or "sometimes". However, in order to evaluate some digestive symptoms in a large number of subjects, it was necessary to make the questionnaire simple. As a result, we obtained a very high response rate (99%). As a second limitation, the subjects in this study were not a true random sample of the population. Approximately 40% of the population in Miyagi prefecture is aged < 40 years, and the majority of the study sample was aged > 40 years. Thus, the age structure of the study subjects was different from that of the true unselected population in Miyagi prefecture. However, previous Japanese population-based studies were performed on subjects who visited the hospital for routine physical examinations and as outpatients^[13-17], and we have to consider the possibility that they are symptomatic, rather than a true random sample of the population. On the other hand, the majority of people in this study were selected according to their age, and they were considered to be less symptomatic than hospital outpatients. Furthermore, we evaluated the clinical symptoms according to each generation. In this regard, this study is considered to be the first to highlight the prevalence of GERD symptoms in a large unselected population of Japanese subjects who are over > 40 years old. This method may have avoided several sources of bias that have tended to occur in previous studies in Japan.

In summary, we clearly described that reflux symptoms are commonly found in the Japanese general population, especially in older women. We conclude that the prevalence of typical GERD symptoms (heartburn) is high, at about 20%, in the Japanese general population, and the frequency is especially high in women in the 60-89 years age group. We found the symptoms of heartburn, dysphagia and

abdominal pain overlapped with each other with high frequency.

COMMENTS

Background

The prevalence of gastroesophageal reflux disease (GERD) symptoms is now increasing in Japan.

Research frontiers

To examine the prevalence of GERD symptoms in a large unselected general population in Japan.

Innovations and breakthroughs

Although there have been many studies on the prevalence of GERD in Japan, all of these have been performed on subjects who visited the hospital as outpatients or for routine physical examination. However, we have to consider the possibility that outpatients are symptomatic, rather than a true random sample of the population. Furthermore, subjects undergoing their annual medical check-up make a periodic visit to the each of the medical institutions for routine physical examination. These subjects may have caused some bias, and subjects who visited the hospital as outpatients or for routine physical examination may not be similar to the general population. We decided to collect data from subjects attending a primary health care institution for an annual medical check-up rather than using data from hospital-based subjects. The majority of people in this study were selected according to their age (> 40 years), and they were considered to be less symptomatic than hospital outpatients.

Applications

This study is considered to be the first to highlight the prevalence of GERD symptoms in a large population of unselected Japanese subjects aged > 40 years old. The prevalence of GERD symptoms among true unselected subjects must continue to be studied in the future.

Terminology

GERD: Gastroesophageal reflux disease.

Peer review

In this study, the authors ascertained the prevalence of gastroesophageal reflux symptoms in an extremely large population in Japan. The study was well performed and the conclusions are clear and compatible with those of previous studies.

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Absence of Na⁺/sugar cotransport activity in Barrett's metaplasia

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Abstract

AIM: To evaluate the presence of Na⁺-dependent, active, sugar transport in Barrett's epithelia as an intestinal biomarker, based on the well-documented, morphological intestinal phenotype of Barrett's esophagus (BE).

METHODS: We examined uptake of the nonmetabolizable glucose analogue, alpha-methyl-D-glucoside (AMG), a substrate for the entire sodium glucose cotransporter (SGLT) family of transport proteins. During upper endoscopy, patients with BE or with uncomplicated gastroesophageal reflux disease (GERD) allowed for duodenal, gastric fundic, and esophageal mucosal biopsies to be taken. Biopsies were incubated in bicarbonate-buffered saline (KRB) containing 0.1 mmol/L ¹⁴C-AMG for 60 min at 20°C. Characterized by abundant SGLT, duodenum served as a positive control while gastric fundus and normal esophagus, known to lack SGLT, served as negative controls.

RESULTS: Duodenal biopsies accumulated 249.84 ± 35.49 (SEM) picomoles AMG/μg DNA (*n* = 12), gastric fundus biopsies 36.20 ± 6.62 (*n* = 12), normal esophagus 12.10 ± 0.59 (*n* = 3) and Barrett's metaplasia 29.79 ± 5.77 (*n* = 8). There was a statistical difference (*P* < 0.01) between biopsies from duodenum and each other biopsy site but there was no statistically significant difference between normal esophagus and BE biopsies. 0.5 mmol/L phlorizin (PZ) inhibited AMG uptake into duodenal mucosa by over 89%, but had no

significant effect on AMG uptake into gastric fundus, normal esophagus, or Barrett's tissue. In the absence of Na⁺ (all Na⁺ salts replaced by Li⁺ salts), AMG uptake in duodenum was decreased by over 90%, while uptake into gastric, esophageal or Barrett's tissue was statistically unaffected.

CONCLUSION: Despite the intestinal enterocyte phenotype of BE, Na⁺-dependent, sugar transport activity is not present in these cells.

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Key words: Barrett's esophagus; Cancer; Biomarker; Sodium glucose cotransporters; Glucose transport; Alpha-methyl-D-glucoside; Phlorizin; Esophagus; Metaplasia

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INTRODUCTION

Esophageal adenocarcinoma, which has increased 4-fold between 1973 and 2002, is escalating at an incidence rate which exceeds that of any other malignancy^[1-3]. Generally detected at a late stage, esophageal adenocarcinoma is extremely lethal, with a 5-year survival rate of just 10%-20%^[4]. Barrett's esophagus (BE), a condition characterized by a columnar, intestinal-like metaplasia of the normally stratified squamous distal esophagus, confers a dramatically increased risk of progression to esophageal adenocarcinoma and serves currently as the most important clinical risk factor for development of such malignancy^[2]. In fact, the risk of esophageal adenocarcinoma in BE patients is 30-120 times that of the general population^[2,5].

BE may be present in 3% of the western hemisphere population and in at least 10% of gastroesophageal reflux disease (GERD) patients^[1]. It is impractical to attempt to identify the BE population by means of endoscopic

surveillance of all GERD patients, considering that there are 30 million Americans with GERD^[6]. Only 5% of patients presenting with esophageal adenocarcinoma are preceded by a BE diagnosis made by upper endoscopy, further illustrating the logistical difficulties in screening a large population by this means^[4]. Thus, it will be valuable to find and establish biomarkers which may be used to identify BE, simply, safely, and inexpensively, in order to appropriately avert esophageal adenocarcinoma.

Current cellular biomarkers of BE include sucrase-isomaltase (SI) and dipeptidyl peptidase IV (DPP), enzymes which are present in about 60% of BE patients^[7]. Cdx2, which encodes an intestine-specific transcription factor, is another possible BE biomarker presently under investigation^[8]. Villin, a cytoskeletal protein related to microvilli, and Ep-CAM, a glandular epithelial glycoprotein are also possible BE biomarkers^[9]. Other potential markers of BE are the deletion of p53 tumor suppressor gene, deletion or hypermethylation of p16, changes in COX-2 expression, and increases in cell proliferation and cell cycling markers like cyclin D1^[2,4]. Two inherent disadvantages of these markers are: (1) each individually will not be found in every Barrett's biopsy and (2) all will require tissue biopsies to be taken (upper endoscopies to be performed).

The heterogeneity of BE is evidenced by the presence of columnar non-goblet cells intermixed with the intestinal-like goblet cells in the metastatic Barrett's population^[7]. The specialized epithelial columnar cells are known to express the small intestinal enzymes, SI and dipeptidyl peptidase, and perhaps represent an "incomplete" stage of intestinal metaplasia^[7]. Additionally, heterogeneity exists with regard to villin and Ep-CAM expression in Barrett's cells, indicating variations in differentiation state within the Barrett's epithelium^[9].

BE, which has a patchy, irregular appearance, typically consists of three distinct cell morphologies: an atrophic gastric fundus type, a junctional gastric cardia type, and an intestinal-like population containing goblet cells and also non-goblet columnar cells (both mucus-containing and pseudo-absorptive in nature)^[5,6,9,10]. Of these three, only the goblet cell population exhibiting an intestinal phenotype is currently definitively associated with increased risk of malignancy and is thus exclusively required for clinical BE diagnosis^[6,8]. The histological detection of mucin, which can be achieved in dramatic fashion by Alcian blue staining defines the 'goblet cell metaplasia' character of true BE^[11].

Of course, histology requires mucosal biopsies, which can be obtained only through upper endoscopy. A major emphasis of current research in BE is to define new biomarkers which may lead to screening techniques not dependent upon endoscopy and biopsies, due to the expense of anesthesia and risk of the procedure.

The intestinal-like phenotype of BE prompted our group to test for the presence of Na⁺-dependent, active, sugar transport activity (the SGLT family of proteins) in BE as a potential biomarker. Glucose is absorbed in the intestine by SGLT through an energy-, concentration- and Na⁺-dependent fashion^[12-14]. SGLT is specific to the differentiated, absorptive epithelial cells in the small intestine, kidney proximal tubule, and a few other epithelial cell types and is absent in the stomach, normal esophagus,

and most other somatic cells^[15-18]. The nonmetabolizable glucose analogue, alpha-methyl-D-glucoside (AMG), is a substrate specific for the sodium-dependent (active) glucose cotransporter (SGLT) family of transport proteins, since a free hydroxyl on carbon 1 is not required for uptake by SGLT^[19]. AMG is, however, a very poor substrate for the more ubiquitous facilitated diffusion transporters of the GLUT family^[20]. Phlorizin (PZ) is a competitive inhibitor of intestinal active glucose transport but is not transported itself by the Na⁺/glucose cotransporter^[21].

MATERIALS AND METHODS

Biopsy acquisition

Under an IRB-approved protocol, patients with and without BE, presenting to the Lankenau Hospital Gastroenterology group for upper endoscopy for screening or diagnostic purposes relating to reflux, gastritis, or dysphagia, provided written informed consent for additional biopsies to be taken during upper endoscopy. The patient population of 23 people included 9 females and 14 males, ranging in age from 47 to 81 years. Ten of the study patients were known to have BE, as confirmed by prior positive Alcian blue staining of endoscopic biopsies.

For simple AMG uptake studies, a total of nine biopsies were taken, three each from duodenum, gastric fundus, and Barrett's metaplasia or normal esophagus. For PZ inhibition and Li⁺ saline/Na⁺ saline studies, a total of eight biopsies were taken from each patient, four each from two of the above listed sites. Duodenum, known to have abundant SGLT, served as a positive control. Gastric fundus and normal esophagus served as negative controls. These tissues are known not to exhibit Na⁺/glucose cotransport activity. By evaluating each BE biopsy individually for uptake of ¹⁴C-AMG, we address the possibility of AMG uptake being expressed in only certain regions of Barrett's epithelium in any individual Barrett's patient.

¹⁴C-AMG uptake studies

For AMG uptake studies, endoscopic biopsies were placed immediately in ice cold, freshly oxygenated bicarbonate-buffered saline (KRB) and transported from the endoscopy procedure room to the laboratory. Because glucose would interfere with AMG uptake, 5 mmol/L sodium acetate was used to replace glucose as a metabolic substrate in the saline. KRB contained 112 mmol/L NaCl, 5 mmol/L KCl, 1.25 mmol/L CaCl₂·2H₂O, 1.1 mmol/L MgCl₂·6H₂O, 2.4 mmol/L Na₂HPO₄, 0.6 mmol/L NaH₂PO₄·H₂O, 25 mmol/L NaHCO₃, 5 mmol/L sodium acetate.

Biopsies were incubated in KRB containing 0.1 mmol/L ¹⁴C-AMG at 20°C for 60 min while being shaken and aerated with 5% CO₂/95% O₂. Methyl-alpha-D [U-¹⁴C] glucopyranoside (AMG) was purchased from Amersham Biosciences (UK). Biopsies were copiously rinsed with 4°C saline (0.154 mol/L NaCl) at the end of the incubation to remove extracellular ¹⁴C-AMG, then a wet weight was recorded followed by addition of biopsy to 0.4 mmol/L perchloric acid for release of intracellular radioactivity, and precipitation (and later measurement) of total DNA by the diphenylamine reaction^[22]. Intracellular ¹⁴C-AMG released from the samples

Table 1 Uptake of AMG by duodenal, gastric fundic, and esophageal mucosal biopsies (mean \pm SE)

	pmoles AMG mg wet wt	pmoles AMG/ μ g DNA
Duodenum	711.53 \pm 121.63 (11)	249.84 \pm 35.49 (12)
Fundus	41.04 \pm 2.35 (12) ^b	36.20 \pm 6.62 (12) ^b
Esophagus	45.52 \pm 3.76 (3) ^a	12.10 \pm 0.59 (3) ^d
Barrett's	50.27 \pm 4.32 (9) ^b	29.79 \pm 5.77 (8) ^b

The number in parenthesis represents the biopsies tested. Three biopsies were taken from each tissue site of each patient. ^b*P* < 0.001 *vs* duodenum; ^a*P* < 0.02 *vs* duodenum; ^d*P* < 0.01 *vs* duodenum (Student's *t*-test). There was no significant difference (*P* > 0.05) between Barrett's esophagus biopsies and biopsies of fundus or normal esophagus.

Table 2 Inhibition of AMG uptake by 0.5 mmol/L phlorizin in duodenal, gastric fundic, and esophageal mucosal biopsies (mean \pm SE)

	Control	Phlorizin
Duodenum	593.34 \pm 120.23 (7)	65.05 \pm 14.41 (7) ^b
Fundus	35.00 \pm 1.82 (8)	30.27 \pm 4.23 (8) ^a
Esophagus	36.73 \pm 4.85 (8)	27.20 \pm 2.63 (8) ^a
Barrett's	53.09 \pm 5.57 (6)	42.66 \pm 4.37 (7) ^a

The number in parenthesis represents the biopsies tested. ^b*P* < 0.001 *vs* matched control (Student's *t*-test); ^a*P* > 0.05 *vs* control.

was quantified by scintillation counting and then normalized per wet weight and per total amount of DNA.

PZ inhibition studies

To confirm the absence of AMG uptake by SGLT, a series of experiments were performed using PZ, a specific, competitive inhibitor of SGLT. PZ dihydrate was purchased from Sigma-Aldrich (St. Louis, MO). For PZ inhibition studies, biopsies were collected in a similar fashion but were pre-incubated in KRB with or without 0.5 mmol/L PZ (and without AMG) for 15 min, while being shaken and aerated with 5% CO₂/95% O₂. After 15 min, 0.1 mmol/L ¹⁴C-AMG \pm 0.5 mmol/L PZ was added to the saline and samples were incubated for an additional 60 min. After the incubation period, samples were similarly rinsed, weighed, and digested for determination of ¹⁴C-AMG uptake per sample wet weight.

Sodium dependence studies

To further confirm a lack of Na⁺/sugar cotransport activity, a series of experiments were performed in which lithium salts replaced sodium salts in the incubation medium. In addition, KRB contained 5 mmol/L lithium acetate in place of sodium acetate. Biopsies were similarly incubated with ¹⁴C-AMG (in KRB with either sodium or lithium salts), rinsed, and weighed for determination of ¹⁴C-AMG uptake per sample wet weight.

RESULTS

Na⁺/sugar cotransport was assayed by measuring uptake of the nonmetabolizable glucose analogue, AMG, a substrate for all three known SGLTs, with K_m values of 0.4 mmol/L

Table 3 Sodium dependence or independence of AMG uptake by duodenal, gastric fundic, and esophageal mucosal biopsies (mean \pm SE)

	Lithium	Sodium
Duodenum	49.31 \pm 5.86 (10) ^b	544.85 \pm 110.20 (10) ^d
Fundus	35.20 \pm 2.73 (8)	41.15 \pm 1.91 (7)
Esophagus	44.83 \pm 4.03 (9)	41.56 \pm 5.07 (9)
Barrett's	40.47 \pm 1.47 (5)	48.03 \pm 11.72 (5)

The number in parentheses represents the biopsies tested. ^b*P* < 0.001, ^d*P* < 0.001 *vs* all other samples. There was no significant difference between lithium and sodium samples.

for SGLT1 and 2 mmol/L for SGLT2 and SGLT3^[23]. AMG is not a substrate for facilitated diffusional transporters (GLUT1, *etc*) and thus cannot easily efflux from the cell^[20], meaning that AMG will accumulate to high intracellular concentrations in cells expressing SGLT. AMG uptake by duodenal biopsy samples was 17-fold greater than gastric biopsies, indicative of the known presence of SGLT in duodenal tissue (Table 1). Dramatically lower AMG uptake in gastric and normal esophageal biopsies correlated with the known absence of SGLT in these tissues. Barrett's biopsies were unable to concentrate AMG to the levels exhibited by duodenal biopsies, suggesting lack of Na⁺-dependent sugar uptake in Barrett's tissue.

To confirm this absence of AMG uptake in Barrett's epithelium, an additional series of experiments was performed using PZ, a competitive inhibitor of sugar uptake specifically by SGLT^[24-27]. AMG uptake by duodenal biopsies was inhibited over 85% by 0.5 mmol/L PZ, consistent with the known presence of Na⁺/sugar cotransport in duodenum (Table 2). AMG uptake in gastric and normal esophageal biopsies was not significantly inhibited by PZ. This same lack of PZ inhibition of AMG uptake into biopsies of Barrett's metaplasia is a further indication of the lack of active sugar transport activity in Barrett's epithelium.

As final verification of the lack of Na⁺/sugar cotransport in Barrett's epithelium, additional experiments were performed in which lithium salts replaced sodium salts in the incubation saline. Intracellular uptake of AMG into duodenal biopsies in Na⁺-free (Li⁺) saline was only 10% of the levels in Na⁺ saline (Table 3). However, the presence of Na⁺ did not significantly affect AMG uptake into gastric, esophageal, or Barrett's tissue biopsies. This lack of Na⁺-dependence of sugar transport further confirms the lack of active sugar transport in Barrett's epithelium.

DISCUSSION

The inability of Barrett's tissue to concentrate AMG, the lack of inhibition by PZ, and the absence of Na⁺-dependence all indicate the absence of an active sugar transport system in BE. Despite the intestinal phenotype ascribed to Barrett's metaplasia, with classic intestinal markers like SI activity and mucin secretion, Barrett's biopsies appear to lack activity associated with SGLT, the active sugar transporter that typifies normal small intestine. The absence of SGLT activity in Barrett's tissue highlights the fact that Barrett's cells are not simply an intestinal cell

type in the distal esophagus, even though Barrett's tissue has other intestinal-like characteristics.

Our results, however, do leave open the possibility, though unlikely, that SGLT is present in the plasma membrane or cells of Barrett's epithelium though it is not functional. Thus, the assays described in this paper were strictly limited to determination of the functional presence of SGLT. Using duodenal biopsies, our group obtained only inconclusive and disappointing results with two commercial SGLT antibodies, therefore not allowing a parallel immunological approach to this question.

Barrett's epithelium develops as a result of damage to the stratified squamous tissue of the distal esophagus by acid and bile from reflux^[5]. Because of the unique, columnar, intestinal-like phenotype of Barrett's epithelium, it is widely accepted that Barrett's epithelium arises from clonal growth of pluripotent stem cells rather than growth from adjacent inflamed esophageal squamous epithelia, as the "de novo" metaplasia theory suggests^[6,9]. The duct cell metaplasia theory posits that Barrett's cells derive from stem cells in esophageal glands that colonize the esophagus when the mucosa is damaged^[4,6]. Finally, the transitional zone metaplasia theory states that, when exposed to bile and acid, the cells of the distal esophagus are replaced by stem cells from the gastroesophageal junction^[5,6]. Whatever the origin, BE is a "successful adaptation" for protection of the esophagus from further reflux damage, saving the individual with reflux from a future of ulceration and strictures, but carrying with it an increased cancer risk^[5].

SGLT is expressed on the apical plasma membrane of absorptive epithelial cells in the small intestine but is not expressed by goblet cells^[17,18,28]. Also, different rates of Na⁺-dependent glucose transport have been observed in mature cells from the villi of intestine and in immature crypt cells, suggesting that differentiation and enterocyte development state may contribute to SGLT expression and affinity levels^[29-31]. One must therefore consider that not all small intestinal cell types possess Na⁺/glucose cotransport and that BE may be reflective of these cell types.

Although SGLT may not serve as a biomarker for BE, our group and others have collected evidence that the transporter may be present in intestinalized gastric mucosa and may potentially indicate metaplastic changes related to gastric adenocarcinoma^[32]. As aforementioned, SGLT is normally not present in the stomach. However, biopsies from a patient with a large, grossly-visible, hyperplastic gastric polyp, exhibited ¹⁴C-AMG uptake that was inhibited by PZ and approximately five times greater than uptake by the patient's normal esophageal biopsies (Data not shown).

In summary, despite the intestinal phenotype ascribed to Barrett's metaplasia with classic intestinal markers like SI activity and mucin secretion, Barrett's biopsies appear to lack activity associated with SGLT, the active sugar transporter present in normal small intestine, and thus cannot serve as a BE biomarker. The absence of SGLT activity in Barrett's biopsies highlights the fact that, although it has other intestinal-like characteristics, Barrett's cells are not simply an intestinal cell type in the distal esophagus, but should rather be treated as a unique entity in future considerations.

COMMENTS

Background

In order to effectively avert esophageal adenocarcinoma, it is necessary to find and establish biomarkers to identify Barrett's esophagus (BE). The purpose of this study was to evaluate the presence of Na⁺-dependent, active, sugar transport in Barrett's epithelia as an intestinal biomarker, based on the well-documented, morphological intestinal phenotype of BE.

Research frontiers

To our knowledge, this is the first paper to investigate Na⁺-dependent, active, sugar transport in Barrett's epithelia as an intestinal biomarker.

Innovations and breakthroughs

Despite the intestinal phenotype ascribed to Barrett's metaplasia with classic intestinal markers like sucrase-isomaltase (SI) activity and mucin secretion, Barrett's biopsies appear to lack activity associated with SGLT, the active sugar transporter present in normal small intestine, and thus cannot serve as a BE biomarker. The absence of SGLT activity in Barrett's biopsies highlights the fact that, although it has other intestinal-like characteristics, Barrett's cells are not simply an intestinal cell type in the distal esophagus, but should rather be treated as a unique entity in future considerations.

Applications

While our studies show a lack of functional SGLT1 protein in BE, it may also be valuable to determine the expression of SGLT1 mRNA in Barrett's tissue by RT-PCR.

Peer review

This nice study set out to assess by functional criteria whether SGLT1 is expressed in Barrett's metaplasia. It well evaluated the presence of Na⁺-dependent, active, sugar transport in Barrett's epithelia as an intestinal biomarker.

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RAPID COMMUNICATION

Des-gamma-carboxy prothrombin as an important prognostic indicator in patients with small hepatocellular carcinoma

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Abstract

AIM: To clarify the effect of a high des-gamma-carboxy prothrombin (DCP) level on the invasiveness and prognosis of small hepatocellular carcinoma.

METHODS: Among 142 consecutive patients with known DCP levels, who underwent hepatectomy because of hepatocellular carcinoma, 85 patients met the criteria for small hepatocellular carcinoma, i.e. one \leq 5 cm sized single tumor or no more than three \leq 3 cm sized tumors.

RESULTS: The overall survival rate of the 142 patients was 92.1% for 1 year, 69.6% for 3 years, and 56.9% for 5 years. Multivariate analysis showed that microscopic vascular invasion ($P = 0.03$) and serum DCP \geq 400 mAU/mL ($P = 0.02$) were independent prognostic factors. In the group of patients who met the criteria for small hepatocellular carcinoma, DCP \geq 400 mAU/mL was found to be an independent prognostic factor for recurrence-free ($P = 0.02$) and overall survival ($P = 0.0005$). In patients who did not meet the criteria, the presence of vascular invasion was an independent factor for recurrence-free ($P = 0.02$) and overall survivals ($P = 0.01$). In 75% of patients with small hepatocellular carcinoma and high DCP levels, recurrence occurred extrahepatically.

CONCLUSION: For small hepatocellular carcinoma, a high preoperative DCP level appears indicative for

tumor recurrence. Because many patients with a high preoperative DCP level develop extrahepatic recurrence, it is necessary to screen the whole body.

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Key words: Small hepatocellular carcinoma; Hepatic resection; Des-gamma-carboxy prothrombin; Vascular invasion; Prognostic factor

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INTRODUCTION

Des-gamma-carboxy prothrombin (DCP) is a tumor marker specific for hepatocellular carcinoma^[1]. It is believed that the elevation of the serum DCP level correlates with the presence of vascular invasion or intrahepatic metastases^[2-8]. Furthermore, DCP has been reported to be an independent prognostic factor for recurrence and survival after hepatic resection^[4,9-13], liver transplantation^[6,14], ablation treatment^[15-17], and transarterial chemoembolization (TAE) treatment^[18]. However, the rate of detectable serum DCP levels in patients with small hepatocellular carcinoma is low^[19-24]. Although methods have been improved^[25,26], sensitivity is still at about 50% for most small cell carcinoma^[27-30]. Thus, almost all reports on the biological nature of DCP and its prognostic value are based on analyses of patients with larger or more advanced tumors with various degrees of hepatic functional reserve. Reports on the relevance of preoperative DCP level as a prognostic marker in small hepatocellular carcinoma patients are rare.

This study thus aimed analysing the predictive value of preoperative serum DCP level on tumor recurrence and prognosis, particularly in hepatocellular carcinoma patients who had undergone liver resection and who met the criteria for small hepatocellular carcinoma^[31], i.e. a single

tumor with all dimensions being 5 cm or less, or no more than three tumors with dimensions of 3 cm or less.

MATERIALS AND METHODS

From a total of 172 consecutive patients who had undergone a first curative hepatic resection for hepatocellular carcinoma at the Hirosaki University Hospital from 1990 to 2004, 142 patients whose preoperative DCP level was measured by a highly-sensitive assay were enrolled. Patients who met the criteria of small hepatocellular carcinoma, i.e. a single tumor with the largest dimension being 5 cm or less, or no more than three tumors with the largest dimension being 3 cm or less, were compared to the others. Sixteen clinical parameters were recorded [age, gender, Child-Pugh score, serum total bilirubin level, serum albumin level, prothrombin activity, 15-min retention rate of indocyanine green (ICG R15), status of hepatitis virus infection, the number of tumors, the largest dimension of the tumor, the degree of tumor differentiation, the presence or absence of macroscopic and microscopic vascular invasion, the extent of tumor (stage), DCP, and alpha-fetoprotein (AFP)] and the predictivity for probability of recurrence and prognosis of survival were evaluated.

DCP was measured by the chemiluminescent immunoassay using a sensitive anti-DCP antibody (Eisai Co., Ltd., Tokyo, Japan), and threshold values were set to 40, 100, 200, and 400 mAU/mL for determining the presence or absence of a positive reaction. Moreover, the extent of tumor was classified according to the stage classification by the Liver Cancer Study Group of Japan^[32].

Statistical analysis

Comparisons between the two groups were carried out by the Chi-square test for categorical data and Student's *t*-test for continuous data. The continuous variables were reported as the mean \pm SD. A Cox proportional hazards model was used to test the significance of 16 parameters as predictors of recurrence-free and overall survivals. Kaplan-Meier method and long rank test were also adapted to compare the effect of these factors on survival. These statistical analyses were performed using the SPSS 11.0 statistical software program. *P* values < 0.05 were considered to be statistically significant.

RESULTS

In this series, 85 of 142 patients met the criteria of small hepatocellular carcinoma, accounting for 60% of the total. Patients' characteristics are given in Table 1. The mean age was 63.0 ± 10.6 years; that of the patients with small hepatocellular carcinoma was significantly higher than that of the patients with greater tumors. No difference was observed between males and females. Concerning Child-Pugh score, Class B was observed more commonly in patients who did not meet the criteria.

Other liver function tests as serum total bilirubin level, albumin level, platelet counts, or ICG R15 were also found to be lower in patients with small hepatocellular carcinoma. A positive status for hepatitis virus, either hepatitis B or C

virus, was more frequent in small hepatocellular carcinoma patients. The largest dimension of the tumor was 2.9 ± 1.2 cm in patients who met the criteria for small hepatocellular carcinoma; it was 7.6 ± 4.6 cm in patients who did not meet the criteria, and lesions > 5 cm in size accounted for more than 70% of the cases in the latter group. Ninety percent of the patients who met the criteria for small hepatocellular carcinoma had a solitary lesion, whereas a significantly larger number of patients outside the criteria had multiple lesions. Pathological evaluation of tumor specimens revealed a significantly higher rate of poorly differentiated tumor cells and a more frequent presence of microscopic vascular invasion in patients who did not meet the criteria for small hepatocellular carcinoma compared to those who did. According to the TNM-Staging by the Liver Cancer Study Group of Japan, approximately 90% of the patients who met the criteria were classified as being in Stage I or II, whereas two-thirds of the patients outside the criteria were classified as being in Stage III or higher. Serum AFP level were not found to be different between the two groups, when threshold was set to 40 ng/mL. However, when a threshold of ≥ 200 ng/mL was chosen, the positive rate among patients outside the criteria was high. On the other hand, the serum DCP showed a lower positive rate among patients who met the criteria for small hepatocellular carcinoma compared to those who did not independently on the threshold value.

The recurrence-free survival and the overall survival of the whole group of patients was 60.3% for 1 year, 29.5% for 3 years, and 13.9% for 5 years, and 92.1% for 1 year, 69.6% for 3 years, and 56.9% for 5 years, respectively. The recurrence-free survival of the patients who met the criteria was 66.2% for 1 year, 34.1% for 3 years, and 16.6% for 5 years, with the overall survival of 97.5% for 1 year, 82.5% for 3 years, and 67.9% for 5 years, which compared particularly well to the recurrence-free survival of patients who did not meet the criteria as 49.9% for 1 year, 20.1% for 3 years, and 7.5% for 5 years ($P = 0.0195$), and the overall survival being 82.8% for 1 year, 46.8% for 3 years, and 36.5% for 5 years ($P < 0.0001$).

An univariate analysis, including all patients revealed the number of tumors, the degree of tissue differentiation, vascular invasion, tumor stage, any of the DCP thresholds, and AFP ≥ 20 ng/mL, as significant prognostic factors for recurrence. Concerning overall survival, the tumor diameter was also a significant prognostic factor (Table 2). Consequently, a multivariate analysis indicated that microscopic vascular invasions ($P = 0.03$) and DCP ≥ 400 mAU/mL ($P = 0.02$) were independent prognostic factors for survival prognosis (Table 3). There was no independent factor reflecting the recurrence-free survival.

The univariate analysis of the 85 patients with small hepatocellular carcinoma showed that DCP of various cut-off values was the most significant prognostic factor (Table 4), and a multivariate analysis showed that DCP ≥ 400 mAU/mL was the only independent prognostic factor for recurrence-free survival (Hazard ratio (HR): 3.32; 95% confidence interval (CI): 1.20-9.17, $P = 0.02$) and overall survival (HR: 1.20; 95% CI: 2.98-50.00, $P = 0.0005$) (Table 5).

On the other hand, a multivariate analysis of the 57 patients outside the criteria showed that microscopic

Table 1 Comparison of demographic and clinical data

Factor		Conforming to the criteria (n = 85)	Outside the criteria (n = 57)	P value
Age (yr)		65.0 ± 8.4	60.1 ± 12.7	0.007
Gender	Male	62 (73%)	44 (77%)	0.57
	Female	23 (27%)	13 (23%)	
Child-Pugh score	Class A	71 (84%)	54 (95%)	0.04
	Class B	14 (16%)	3 (5%)	
	Class C	0	0	
Total bilirubin (mg/dL)		0.90 ± 0.55	0.74 ± 0.32	0.04
Albumin (g/dL)	< 1	42 (49%)	41 (72%)	0.008
	≥ 1	43 (51%)	16 (28%)	
Prothrombin time (%)	≤ 3.5	39 (46%)	14 (25%)	0.01
	> 3.5	46 (54%)	43 (75%)	
Platelet count (× 10 ⁴ /mm ³)	≤ 80	83.0 ± 15.2	85.9 ± 17.6	0.30
	> 80	35 (41%)	25 (44%)	
ICG R15 (%)	< 10	50 (59%)	32 (56%)	< 0.0001
	≥ 10	11.8 ± 6.3	17.5 ± 8.6	
Hepatitis virus	< 15	19.8 ± 9.9	14.1 ± 1.2	0.0005
	≥ 15	26 (31%)	35 (61%)	
Tumor number	C positive	59 (69%)	22 (39%)	< 0.0001
	B positive	68 (80%)	26 (46%)	
Tumor size (cm)	Single	8 (10%)	15 (26%)	0.008
	Multiple	77 (91%)	29 (51%)	
Histology	< 3	8 (9%)	28 (49%)	< 0.0001
	3-5	2.9 ± 1.2	7.6 ± 4.6	
	> 5	52 (61%)	5 (9%)	
Vascular invasion	≤ 3	33 (39%)	11 (19%)	< 0.0001
	> 3	0	41 (72%)	
TNM Staging by the LCSCG ¹	Well or moderately differentiated	68 (87%)	39 (71%)	0.02
	Poorly differentiated	10 (13%)	16 (29%)	
AFP (ng/mL)	Macroscopically positive	0	5 (8.8%)	0.005
	Microscopically positive	8 (10%)	18 (32%)	
DCP (mAU/mL)	I / II / III / IV-A	25/51/8/1	2/17/25/13	< 0.0001
	I + II	76 (89%)	19 (33%)	
	III + IV-A	9 (11%)	38 (67%)	
AFP (ng/mL)				< 0.0001
AFP (ng/mL)				0.15
AFP (ng/mL)				0.98
AFP (ng/mL)				0.13
AFP (ng/mL)				0.003
AFP (ng/mL)				0.0002
AFP (ng/mL)				0.02
AFP (ng/mL)				0.007
AFP (ng/mL)				0.0001
AFP (ng/mL)				< 0.0001
AFP (ng/mL)				< 0.0001

¹Liver Cancer Study Group of Japan.

vascular invasion was the only independent prognostic factor for recurrence-free survival (HR: 2.97; 95% CI: 1.17-7.58, $P = 0.02$) and overall survival (HR: 3.92; 95% CI: 1.38-11.24, $P = 0.01$) (Table 6).

By performing a Kaplan-Meier analysis of small hepatocellular carcinoma patients, the period of recurrence-free survival was found to be significantly shorter in the group of patients with DCP levels ≥ 400 mAU/mL ($P = 0.02$), and more than 70% of the patients experienced some recurrence within 1 year (Figure 1A). Tumor recurrence after surgery occurred within the liver in 95% of patients with DCP levels < 400 mAU/mL, but extrahepatically in 75% of patients with DCP levels ≥ 400 mAU/mL ($P < 0.0001$). At the time of tumor recurrence, an elevation of DCP levels was observed among all patients. Most of the patients with tumor recurrence received TAE, local ablation

therapy, or a second hepatectomy, but the overall survival also significantly decreased for the group of DCP ≥ 400 mAU/mL ($P < 0.0001$) (Figure 1B).

DISCUSSION

In this series, a high preoperative level of DCP was the only prognostic indicator for recurrence and poor prognosis in patients who underwent hepatectomy for a small hepatocellular carcinoma. Presence of microscopic vascular invasion, on the other hand, was the independent predictor of poor prognosis of both recurrence-free and overall survivals in more advanced hepatic carcinomas. Thus, different results were obtained for the prognostic factors, depending on disease progression.

There are reports that a high DCP level correlates

Table 2 Univariate analysis for recurrence-free and overall survivals in 142 patients undergoing hepatectomy for hepatocellular carcinoma

Factor	Covariate (n)	Reference (n)	Recurrence-free survival			Overall survival		
			HR	95% CI	P	HR	95% CI	P
Gender	Female (36)	Male (106)	1.00	0.63-1.58	0.99	1.26	0.73-2.18	0.42
Child-Pugh score	B (17)	A (125)	0.77	0.42-1.42	0.4	0.87	0.42-1.84	0.72
Total bilirubin (mg/dL)	≥ 1 (59)	< 1 (83)	1.14	0.76-1.70	0.53	1.13	0.68-1.87	0.64
Albumin (g/dL)	≤ 3.5 (53)	> 3.5 (89)	0.93	0.62-1.40	0.74	1.13	0.69-1.86	0.62
Prothrombin time (%)	≤ 80 (60)	> 80 (82)	1.34	0.89-2.01	0.16	1.31	0.79-2.16	0.30
Platelet (× 10 ³ /mm ³)	< 10 (48)	≥ 10 (94)	0.92	0.61-1.40	0.71	0.82	0.49-1.39	0.47
ICG R15	≥ 15% (81)	< 15% (61)	0.94	0.63-1.40	0.76	0.79	0.48-1.31	0.36
Hepatitis C virus	Positive (94)	Negative (48)	1.24	0.80-1.93	0.33	0.87	0.52-1.48	0.62
Hepatitis B virus	Positive (23)	Negative (118)	0.99	0.58-1.70	0.97	1.39	0.74-2.62	0.30
Number of tumor	Multiple (36)	Single (106)	1.70	1.07-2.71	0.02	2.08	1.22-3.54	0.007
Size of tumor (cm)	> 5 (85)	≤ 5 (57)	1.37	0.86-2.17	0.18	2.00	1.08-3.70	0.03
Histology	Poor ¹ (26)	Well-mod ² (107)	1.70	1.01-2.85	0.04	1.81	0.90-3.65	0.10
Vascular invasion	Present (20)	Absent (113)	1.96	1.19-3.23	0.008	2.36	1.32-4.20	0.004
Stage by LCSGJ ³	III + IV (47)	I + II (85)	1.99	1.29-3.06	0.001	2.86	1.71-4.76	< 0.0001
AFP (ng/mL)	≥ 20 (87)	< 20 (55)	1.71	1.11-2.62	0.01	1.90	1.09-3.33	0.02
	≥ 100 (54)	< 100 (88)	1.19	0.79-1.80	0.41	1.43	0.86-2.39	0.16
	≥ 200 (40)	< 200 (102)	1.06	0.68-1.66	0.07	1.25	0.73-2.16	0.41
	≥ 400 (34)	< 400 (108)	1.24	0.78-1.96	0.36	1.59	0.91-2.79	0.10
DCP (mAU/mL)	≥ 40 (88)	< 40 (54)	1.75	1.14-2.70	0.01	1.70	1.00-2.90	0.05
	≥ 100 (62)	< 100 (80)	1.86	1.23-2.80	0.003	1.82	1.10-3.00	0.02
	≥ 200 (46)	< 200 (96)	1.75	1.14-2.70	0.01	3.03	1.79-5.15	< 0.0001
	≥ 400 (38)	< 400 (104)	1.84	1.17-2.89	0.008	2.98	1.73-5.13	< 0.0001

n: Number of patients; HR: Hazard ratio; CI: Confidence interval. ¹Poorly differentiated; ²Well or moderately differentiated hepatocellular carcinoma; ³LCSGJ: Liver Cancer Study Group of Japan.

Table 3 Multivariate analysis for recurrence-free and overall survivals in 142 patients undergoing hepatectomy for hepatocellular carcinoma

Factor	Covariate (n)	Reference (n)	Recurrence-free survival			Overall survival		
			HR	95%CI	P	HR	95% CI	P
Number of tumor	Multiple (36)	Single (106)	0.93	0.39-2.23	0.87	0.82	0.29-2.35	0.71
Size of tumor (cm)	> 5 (85)	≤ 5 (57)	0.93	0.51-1.82	0.82	1.00	0.41-2.42	1.00
Histology	Poor ¹ (26)	Well-mod ² (107)	1.14	0.63-2.05	0.67	0.73	0.33-1.61	0.44
Vascular invasion	Present (20)	Absent (113)	1.68	0.99-2.86	0.05	2.02	1.07-3.83	0.03
Stage by LCSGJ ³	III+IV (47)	I + II (85)	1.72	0.73-2.69	0.22	2.24	0.78-6.45	0.13
AFP (ng/mL)	≥ 20 (87)	< 20 (55)	1.36	0.83-2.22	0.23	1.55	0.81-2.97	0.18
DCP (mAU/mL)	≥ 400 (38)	< 400 (104)	1.45	0.78-2.69	0.24	2.44	1.15-5.21	0.02

n: Number of patients; HR: Hazard ratio; CI: Confidence interval. ¹Poorly differentiated; ²Well or moderately differentiated hepatocellular carcinoma; ³LCSGJ: Liver Cancer Study Group of Japan.

with invasiveness and the metastasizing property of carcinoma^[2-8]. Shirabe *et al*^[8] reported that the preoperative DCP level, tumor diameter, and histologic differentiation correlated with the presence or absence of microscopic vascular invasion in a study on 218 patients who had undergone hepatic resection. Sakon *et al*^[2], Suehiro *et al*^[4], Grazi *et al*^[3], Sugimoto *et al*^[5], and Nanashima *et al*^[13] also reported that a high preoperative DCP level correlated with the presence of microscopic vascular invasion in patients undergoing hepatectomy. Shimada *et al*^[6] reported in a study on 40 patients who had undergone a living donor liver transplantation DCP levels ≥ 300 mAU/mL to be correlated with the presence of microscopic vascular invasion and DCP thus to be a poor prognostic factor.

However, the above reports regarding the invasive character of carcinoma among patients with a high level of DCP were based on the analyses of those with various

cancer stages, including more advanced tumors and those with various degrees of liver cirrhosis. There are only a few reports on patients who had undergone a resection for small hepatocellular carcinoma, because vascular invasion or intrahepatic metastases are seldom seen in this group with earlier stage.

It has been reported that the rate of detectable levels of DCP is higher in patients with larger tumors^[19]. On the other hand, the positive rate of DCP detectability is low for small hepatocellular carcinoma^[19-24]. Okuda *et al*^[24] reported that it was 81.3% with a tumor diameter of ≥ 3 cm, while it was 30.4% for ≤ 2 cm. Sassa *et al*^[27] reported that it was 44.3% for ≤ 2 cm. An assay that employs higher DCP diagnostic sensitivity has been introduced^[25,26], but the diagnostic sensitivity for small hepatocellular carcinoma still remains at about 50%^[27-30]. Thus, most reports on biological properties of DCP positive hepatic

Table 4 Univariate analysis for recurrence-free and overall survivals in 85 patients who met the criteria

Factor	Covariate (n)	Reference (n)	Recurrence-free survival			Overall survival		
			HR	95% CI	P	HR	95% CI	P
Gender	Female (23)	Male (62)	0.81	0.44-1.48	0.48	0.91	0.43-1.94	0.81
Child-Pugh score	B (14)	A (71)	0.83	0.43-1.61	0.58	1.06	0.46-2.43	0.89
Total bilirubin (mg/dL)	≥ 1 (43)	< 1 (42)	1.37	0.82-2.30	0.23	1.77	0.83-3.79	0.14
Albumin (mg/dL)	≤ 3.5 (39)	> 3.5 (46)	0.92	0.55-1.52	0.74	0.56	0.28-1.09	0.09
Prothrombin time (%)	≤ 80 (35)	> 80 (50)	1.25	0.75-2.08	0.40	0.82	0.42-1.61	0.56
Platelet (× 10 ³ /mm ³)	< 10 (35)	≥ 10 (50)	1.05	0.63-1.76	0.84	0.98	0.50-1.92	0.95
ICG R15	≥ 15% (59)	< 15% (26)	1.37	0.79-2.38	0.27	1.51	0.70-3.26	0.29
Hepatitis C virus	Positive (68)	Negative (17)	1.42	0.75-2.70	0.28	1.46	0.60-3.52	0.40
Hepatitis B virus	Positive (8)	Negative (76)	0.63	0.25-1.57	0.32	0.56	0.13-2.34	0.43
Number of tumor	Multiple (8)	Single (77)	1.12	0.48-2.61	0.79	1.29	0.50-3.36	0.60
Size of tumor (cm)	> 3 (33)	≤ 3 (52)	1.08	0.64-1.81	0.78	1.27	0.63-2.56	0.50
Histology	Poor ¹ (10)	Well-mod ² (68)	1.32	0.56-3.11	0.53	3.03	0.89-10.29	0.08
Vascular invasion	Present (8)	Absent (75)	0.96	0.38-2.06	0.77	0.88	0.31-2.54	0.82
Stage by LCSGJ ³	III + IV (9)	I + II (76)	1.41	0.64-3.12	0.39	1.80	0.74-4.35	0.20
AFP (ng/mL)	≥ 20 (52)	< 20 (33)	1.47	0.87-2.49	0.15	1.50	0.75-3.02	0.25
DCP (mAU/mL)	≥ 100 (28)	< 100 (57)	0.77	0.42-1.39	0.39	0.80	0.36-1.75	0.57
	≥ 200 (16)	< 200 (69)	0.53	0.25-1.13	0.10	0.50	0.18-1.42	0.20
	≥ 400 (11)	< 400 (74)	0.57	0.23-1.43	0.23	0.53	0.13-2.21	0.38
	≥ 40 (45)	< 40 (40)	1.73	1.03-2.92	0.04	1.46	0.75-2.82	0.27
	≥ 100 (26)	< 100 (59)	1.67	0.96-2.91	0.07	1.71	0.83-3.50	0.14
	≥ 200 (16)	< 200 (69)	1.69	0.84-3.40	0.14	5.81	2.56-13.16	< 0.0001
	≥ 400 (11)	< 400 (74)	2.41	1.12-5.18	0.02	5.71	2.38-13.70	< 0.0001

n: Number of patients; HR: Hazard ratio; CI: Confidence interval. ¹Poorly differentiated; ²Well or moderately differentiated hepatocellular carcinoma; ³LCSGJ: Liver Cancer Study Group of Japan.

Table 5 Multivariate analysis for recurrence-free and overall survivals in 85 patients who met the criteria

Factor	Covariate (n)	Reference (n)	Recurrence-free survival			Overall survival		
			HR	95% CI	P	HR	95% CI	P
Number of tumor	Multiple (8)	Single (77)	0.66	0.06-7.45	0.74	0.46	0.006-35.08	0.72
Histology	Poor ¹ (10)	Well-mod ² (68)	0.70	0.24-2.01	0.51	0.37	0.07-2.12	0.27
Vascular invasion	Present (8)	Absent (75)	1.04	0.44-2.46	0.93	0.96	0.31-2.99	0.95
Stage by LCSGJ ³	III + IV (9)	I + II (76)	1.90	0.17-21.28	0.60	3.97	0.05-333.33	0.53
AFP (ng/mL)	≥ 20 (52)	< 20 (33)	1.28	0.71-2.29	0.41	1.20	0.54-2.66	0.66
DCP (mAU/mL)	≥ 400 (11)	< 400 (74)	3.32	1.20-9.17	0.02	1.20	2.98-50.00	0.0005

n: Number of patients; HR: Hazard ratio; CI: Confidence interval. ¹Poorly differentiated; ²Well or moderately differentiated hepatocellular carcinoma; ³LCSGJ: Liver Cancer Study Group of Japan.

Table 6 Multivariate analysis for recurrence-free and overall survivals in 57 patients who did not meet the criteria

Factor	Covariate (n)	Reference (n)	Recurrence-free survival			Overall survival		
			HR	95% CI	P	HR	95% CI	P
Gender	Female (13)	Male (44)	1.35	0.56-3.25	0.50	2.35	0.90-6.17	0.08
Number of tumor	Multiple (28)	Single (29)	0.84	0.32-2.25	0.73	0.72	0.24-2.20	0.57
Histology	Poor ¹ (16)	Well-mod ² (39)	1.14	0.47-2.76	0.76	0.61	0.21-1.73	0.35
Vascular invasion	Present (18)	Absent (38)	2.97	1.17-7.58	0.02	3.92	1.38-11.24	0.01
Stage by LCSGJ ³	III + IV (38)	I + II (19)	1.65	0.57-4.78	0.36	1.92	0.52-7.09	0.33
AFP (ng/mL)	≥ 20 (35)	< 20 (22)	1.58	0.59-4.22	0.36	1.85	0.52-6.54	0.34
DCP (mAU/mL)	≥ 400 (27)	< 400 (30)	0.79	0.35-1.79	0.58	2.29	0.35-2.29	0.83

n: Number of patients; HR: Hazard ratio; CI: Confidence interval. ¹Poorly differentiated; ²Well or moderately differentiated hepatocellular carcinoma; ³LCSGJ: Liver Cancer Study Group of Japan.

carcinoma are based on more advanced diseases^[7].

Many reports state high DCP levels to be of poor prognostic value. It has been shown that patients with high DCP and high AFP levels show poor prognostic factors^[4,6,10-12,14-18]. Thus, a prognostic staging system in

which DCP has been incorporated is being suggested. From an analysis of 141 patients Kawakita *et al*^[15] concluded DCP ≥ 100 mAU/mL to be a prognostic factor, and the authors suggested a stage classification in which DCP ≥ 100 mAU/mL should be incorporated into

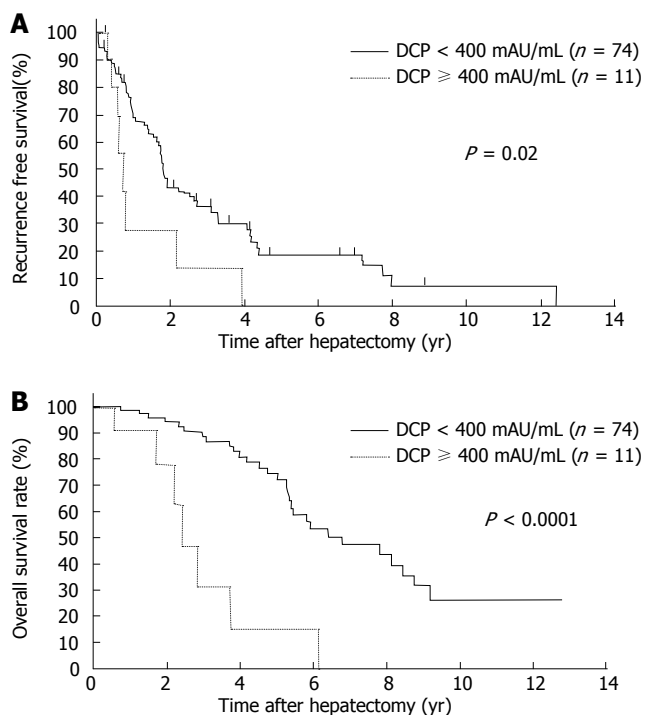


Figure 1 Postoperative recurrence-free (A) and overall (B) survival of patients with small hepatocellular carcinoma. In 85 patients who met the criteria for small hepatocellular carcinoma, the period of recurrence-free survival was significantly shorter in patients with DCP ≥ 400 mAU/mL than in those with DCP < 400 mAU/mL ($P = 0.02$). More than 70% of recurrences occurred within a year (A). Overall survival was also significantly shorter in patients with DCP ≥ 400 mAU/mL (B).

the CLIP score. Nanashima *et al*^[13] concluded that DCP ≥ 400 mAU/mL was a poor prognostic factor and suggested modified CLIP scoring. Moreover, Omagari *et al*^[16] suggested the SLiDe score combined with stages and liver damage, and Toyoda *et al*^[17] suggested the BALAD score that can predict the prognosis only by measuring bilirubin, albumin, AFP-L3, AFP, and DCP using preoperative serum samples, according to an analysis of 2600 patients.

In this study, we used the criteria of small hepatocellular carcinoma “a 5-cm single tumor or no more than three 3-cm tumors” as suggested by Mazzaferro *et al*^[31]. These criteria are internationally accepted as inclusion criteria for liver transplantation for unresectable small hepatocellular carcinoma, because they reflect a restriction of the tumor to the liver^[33].

The univariate analyses of 16 clinical parameters in all patients indicated that the number of tumors, the tumor diameter, the degree of histologic differentiation, vascular invasion, the tumor staging, serum AFP level, and DCP level to be significant prognostic factors. These results are in accordance with previous reports^[3,8,10,12,15-19,34-36]. However, limiting this to small hepatocellular carcinoma, DCP alone is the independent prognostic factor for both tumor recurrence and patients’ survival, while presence of microscopic vascular invasion is a prognostic factor in patients outside the criteria. Regarding the mechanism of these different results, we assume the following. “Specifically, a high DCP level constitutes a risk factor of microscopic vascular invasion and in small hepatocellular carcinoma, DCP shows positive before the development

of microscopic vascular invasion and becomes an independent prognostic factor. As a tumor becomes larger, the frequency of the detection of microscopic vascular invasion increases and the independence of DCP disappears, thus showing a stronger correlation with the prognosis than does DCP.”

DCP is an abnormal prothrombin that is produced by under-carboxylation of normal prothrombin^[37]. Suzuki *et al*^[38] reported that DCP exerts a mitogenic effect on hepatocellular carcinoma cells *via* a Met-Janus kinase 1-STAT3 signaling pathway. On the other hand, it has been demonstrated that the antiproliferative effect of vitamin K on hepatic carcinoma is not due to a depressed production of DCP, but rather caused by protein kinase A^[39]. Regarding the above, the mechanism of the antiproliferative effect of DCP on hepatic carcinoma is still unknown. However, in this study, it was clarified that a high DCP level is an important prognostic factor for recurrence, even in a condition in which small hepatocellular carcinoma before the histological invasion of carcinoma such as vascular invasion becomes obvious. This result corresponds to reports by Koike *et al*^[40] and Hagiwara *et al*^[34] showing that patients who have a high DCP level can expect the expression of future vascular invasion. Moreover, in many of our patients tumor recurred extrahepatically early after resection. Regarding the precise mechanism of DCP on hepatic carcinoma development, it would be necessary to study its effect on the initiation of vascular invasion and on proliferative activity.

COMMENTS

Background

Des-gamma-carboxy prothrombin (DCP) is a marker specific for hepatocellular carcinoma, which has been reported to correlate with the presence of vascular invasion. These reports, however, are based mostly on analyses on advanced carcinoma.

Research frontiers

In this study, the authors retrospectively reviewed the prognostic factors in patients undergoing a first hepatectomy for hepatocellular carcinoma, with special reference to the effect of a high DCP level on the prognosis of small hepatocellular carcinoma.

Innovations and breakthroughs

Preoperative DCP was found to have a different prognostic impact in small hepatocellular carcinoma compared to more advanced tumors. DCP ≥ 400 mAU/mL was an independent prognostic factor for recurrence-free and overall survivals in patients with small hepatocellular carcinoma.

Applications

Preoperative DCP could be integrated into the prognostic scoring, staging and inclusion criteria for hepatectomy, and liver transplantation in patients with small hepatocellular carcinoma.

Peer review

The paper with its scientific and innovative contents as well as readability reflects the advanced level of the clinical research in gastroenterology both at home and abroad.

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RAPID COMMUNICATION

MK615 inhibits pancreatic cancer cell growth by dual inhibition of Aurora A and B kinases

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Abstract

AIM: To investigate the anti-neoplastic effect of MK615, an anti-neoplastic compound isolated from Japanese apricot, against human pancreatic cancer cells *in vitro*.

METHODS: Three human pancreatic cancer cell lines PANC-1, PK-1, and PK45H were cultured with MK615 at concentrations of 600, 300, 150, and 0 $\mu\text{g/mL}$. Growth inhibition was evaluated by cell proliferation assay, and killing activity was determined by lactate dehydrogenase (LDH) assay. Expression of Aurora A and B kinases was detected by real-time polymerase chain reaction (PCR) and Western blotting. Cell cycle stages were evaluated by flow cytometry.

RESULTS: The growth inhibitory rates of MK615 at 150, 300, and 600 $\mu\text{g/mL}$ were $2.3\% \pm 0.9\%$, $8.9\% \pm 3.2\%$ and $67.1\% \pm 8.1\%$ on PANC1 cells, $1.3\% \pm 0.3\%$, $8.7\% \pm 4.1\%$ and $45.7 \pm 7.6\%$ on PK1 cells, and $1.2 \pm 0.8\%$, $9.1\% \pm 2.1\%$ and $52.1\% \pm 5.5\%$ on PK45H cells, respectively ($P < 0.05$). The percentage cytotoxicities of MK615 at 0, 150, 300, and 600 $\mu\text{g/mL}$ were $19.6\% \pm 1.3\%$, $26.7\% \pm 1.8\%$, $25.5\% \pm 0.9\%$ and $26.4\% \pm 0.9\%$ in PANC1 cells, $19.7\% \pm 1.3\%$, $24.7\% \pm 0.8\%$, $25.9\% \pm 0.9\%$ and $29.9\% \pm 1.1\%$ in PK1 cells, and $28.0\% \pm 0.9\%$, $31.2\% \pm 0.9\%$, $30.4\% \pm 1.1\%$ and $35.3 \pm 1.0\%$ in PK45H cells, respectively ($P < 0.05$). Real-time PCR and Western blotting showed that MK615 dually inhibited the expression of Aurora A and B kinases. Cell cycle analysis revealed that MK615 increased the population of cells in G2/M phase.

CONCLUSION: MK615 exerts an anti-neoplastic effect on human pancreatic cancer cells *in vitro* by dual inhibition of Aurora A and B kinases.

INTRODUCTION

MK615, an extract from the Japanese apricot, *Prunus mume* Siebold et Zuccarini (*ume* in Japanese), contains several triterpenoids and has been shown to exert an anti-neoplastic effect against human cancers. Previous studies have revealed that MK615 has anti-neoplastic effects against gastric cancer^[1], breast cancer^[2], hepatocellular carcinoma^[3], and colon cancer^[4]. The mechanisms responsible for the anti-neoplastic effect of MK615 include induction of apoptosis^[1,2] and autophagy^[4], and suppression of Aurora A kinase^[3] in cancer cells. However, the entire mechanisms of the anti-neoplastic effects of MK615 have not been elucidated.

In the present study, we cultured human pancreatic cancer cell lines in the absence and presence of MK615 for the first time, and found that MK615 had an anti-neoplastic effect that was exerted by dual inhibition of Aurora A and B kinases.

MATERIALS AND METHODS

Reagents

MK615 was provided by Japan Apricot Co., Ltd. (Gunma, Japan). MK615 is derived from Japanese apricot fruit^[1].

Cell culture

Three human pancreatic cancer cell lines PANC-1 (PANC1), PK-1 (PK1) and PK-45H (PK45H) were obtained from the Cell Resource Center for Biomedical Research, Institute of Development, Aging and Cancer, Tohoku University (Miyagi, Japan). The cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 100 mL/L

heat-inactivated fetal calf serum (FCS) and incubated at 37°C in a humidified atmosphere consisting of 50 mL/L CO₂ in air.

Cell proliferation assay

Cells were plated at 5×10^3 /well in 96-well plates in DMEM containing 100 mL/L FCS and cultured with or without MK615 at concentrations of 600, 300, or 150 µg/mL. For the negative control wells, cells were cultured with 10 mL/L DMSO alone. After 48 h, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTT; 5 g/L) was added to each well and the cells were incubated for 4 h. Then according to the manufacturer's recommendation, 100 µL of solubilization solution/stop mix was added and the plates were left to stand for 60 min. The absorbances at 570 nm (A_{570}) and 630 nm (A_{630}) were then measured with an enzyme-linked immunoassay (ELISA) reader. The actual counts were calculated by subtracting A_{570} from A_{630} . Each assay was performed in triplicate and the average absorbance was calculated. The growth inhibition rate was calculated using the ratio of absorbance at each drug concentration relative to the absorbance without drug.

LDH assay

Lactate dehydrogenase (LDH) assay was performed using the CytoTox 96® Non-Radioactive Cytotoxicity Assay (Promega, Madison, WI). Briefly, cells were plated at 5×10^3 /well in 96-well plates in DMEM containing 100 mL/L FCS. Cells were cultured with or without MK615 at concentrations of 600, 300, or 150 µg/mL. For the negative control wells, cells were cultured with 10 mL/L DMSO alone. The medium was removed and 100 µL of CytoTox-ONE™ Reagent was added. The cells were then incubated at 22°C for 10 min and 50 µL of stop solution was added. The plates were shaken for 10 s and the absorbances at 560 nm (A_{560}) and 590 nm (A_{590}) were measured with an ELISA reader. To obtain the maximum LDH release, 10 µL of lysis solution (10 ×) was added to the positive control wells 45 min prior to harvest. After 48 h, 50 µL of supernatant was transferred into a fresh 96-well plate and incubated with 50 µL of substrate mix for 30 min at room temperature. Fifty microliters of stop solution was added, and the absorbance at 490 nm (A_{490}) was measured with an ELISA reader. Each assay was performed in triplicate and the average absorbance was calculated. The percentage cytotoxicity was calculated using the formula: Percentage cytotoxicity = $100 \times (\text{Experimental} - \text{Culture medium background}) / (\text{Maximum LDH release} - \text{Culture medium and lysis solution background})$.

Real-time PCR

The total RNA of each cell line was isolated using a Total RNA Isolation kit (MACHEREY-NAGEL, Düren, Germany). Reverse transcription reactions were performed using a Rever Tra Ace α-First Strand cDNA Synthesis kit (TOYOBO, Osaka, Japan). Briefly, 1 µg of total RNA, oligo dT-primer, and dNTPs were incubated at 65°C for

5 min, followed by addition of 10 µL of cDNA synthesis mixture and further incubation at 50°C for 50 min. The reaction was terminated by adding 1 µL of RNaseH and incubating the mixture at 37°C for 20 min.

Real-time PCR was performed with an ABI Prism 7700 Sequence Detector (Applied Biosystems, Warrington, UK). The PCR reaction was carried out in a final volume of 2 µL of cDNA, 12.5 µL of 2 × SYBR Green (Applied Biosystems), 0.5 µL of 25 nmol/L sense and antisense primers, and H₂O up to 25 µL. The PCR conditions consisted of 40 amplification cycles at 95°C for 30 s and 60°C for 30 s. The sequences of the primers were as follows: GAPDH, 5'-CCACCCAGAAGACTGTGGAT-3' (sense) and 5'-TTCAGCTCAGGGATGACCTT-3' (anti-sense); Aurora A, 5'-TTGGAATATGCACCACTTGGA-3' (sense) and 5'-ACTGACCACCCAAATCTGC-3' (anti-sense); and Aurora B, 5'-GGGAGAGCTGAAGATTGCTG-3' (sense) and 5'-GGCGATAGGTCTCGTTGTGT-3' (anti-sense). The level of expression was calculated using the formula: Relative expression (t) = (Copy number of target molecule / Copy number of GAPDH) × 1000. Samples were assayed in triplicate. Means and standard deviations were calculated from the data obtained. The *t* value was calculated from the mean of three different assays.

Western blotting

Anti-Aurora A and Aurora B, nuclear factor-κB (NF-κB), and phosphorylated NFκB at serine residue 536 [pNF-κB (ser536)] antibodies were purchased from Cell Signaling Technology (Beverly, MA). Cells ($5-10 \times 10^6$) were cultured with MK615 (300 µg/mL) for 48 h, washed twice with cold PBS, lysed with 200 µL of 5 g/L SDS, and centrifuged at $10\,000 \times g$. The supernatants were adjusted to contain equal amounts of protein by dilution, using a BCA Protein Assay kit (PIERCE, Rockford, IL). Samples (20 µg protein) were run on 125 g/L SDS-PAGE with 10% gel and electroblotted onto PVDF membranes. The blots were blocked for 1 h with 50 g/L nonfat milk powder and 1 mL/L Tween 20 in Tris-NaCl, followed by overnight incubation with primary antibody (dilution 1:1000) at 4°C. After extensive washing, the blots were incubated with the secondary horseradish-peroxidase-conjugated antibody (dilution 1:2000) for 2 h at room temperature. Immunoreactive bands were visualized by using an enhanced chemiluminescence detection system (Amersham Life Sciences, Arlington Heights, IL).

Cell cycle analysis

Cells were cultured with 300 µg/mL MK615 for 48 h, then collected by trypsinization. According to the manufacturer's instructions, cell cycle analysis was performed with a Cycle TEST PLUS DNA Reagent kit (Becton-Dickinson, Mountain View, CA) on a FACScaliber (Becton-Dickinson). Results were analyzed with CellFIT software.

Statistical analysis

For the MTT assay, LDH assay, and real-time PCR, Student's *t* test (two-sided) was used to compare the data obtained from MK615-treated and MK615-untreated groups. A *P* value less than 0.05 was considered statistically significant.

Table 1 Cell cycle analysis showing MK615 treatment increased the cell population in G2-M phase in all three pancreatic cancer cell lines.

	MK615	G0-G1	S	G2-M
PANC1	(-)	68.1	24.2	7.8
	(+)	71.6	15.4	13.0
PK1	(-)	55.9	42.2	1.8
	(+)	68.8	26.0	5.2
PK45H	(-)	56.0	21.1	22.9
	(+)	59.0	11.7	29.3

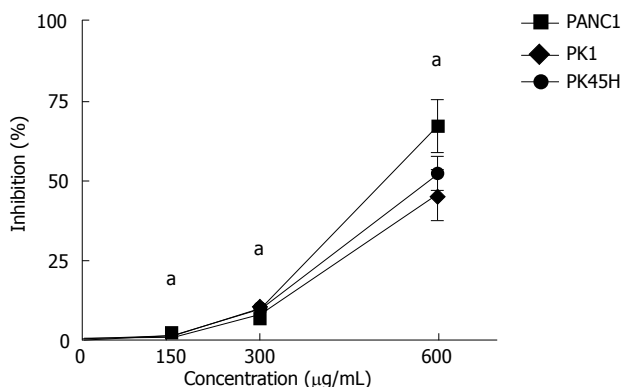


Figure 1 Growth inhibition of pancreatic cancer cells by MK615. MK615 significantly inhibited the growth of PANC1, PK1, and PK45H cells in a dose-dependent manner ($^*P < 0.05$). Cells were cultured for 48 h. The assay was carried out in triplicate.

RESULTS

MK615 inhibited growth of the three pancreatic cancer cell lines (Figure 1). The growth inhibitory rates of MK615 at 150, 300 and 600 $\mu\text{g/mL}$ were $2.3\% \pm 0.9\%$, $8.9\% \pm 3.2\%$ and $67.1\% \pm 8.1\%$ on PANC1 cells, $1.3\% \pm 0.3\%$, $8.7\% \pm 4.1\%$ and $45.7\% \pm 7.6\%$ on PK1 cells, and $1.2\% \pm 0.8\%$, $9.1\% \pm 2.1\%$ and $52.1\% \pm 5.5\%$ on PK45H cells, respectively. The growth inhibition induced by MK615 at all concentrations was significantly higher than that at 0 $\mu\text{g/mL}$ ($P < 0.05$).

Cytotoxicity of MK615 against pancreatic cancer cell lines was evaluated by LDH assay (Figure 2). The percent specific lysis by MK615 at 0, 150, 300, and 600 $\mu\text{g/mL}$ was $19.6\% \pm 1.3\%$, $26.7\% \pm 1.8\%$, $25.5\% \pm 0.9\%$ and $26.4\% \pm 0.9\%$ in PANC1 cells, $19.7\% \pm 1.3\%$, $24.7\% \pm 0.8\%$, $25.9\% \pm 0.9\%$ and $29.9\% \pm 1.1\%$ in PK1 cells, and $28.0\% \pm 0.9\%$, $31.2\% \pm 0.9\%$, $30.4\% \pm 1.1\%$ and $35.3\% \pm 1.0\%$ in PK45H cells, respectively. The percent specific lysis induced by MK615 at all concentrations was significantly higher than that at 0 $\mu\text{g/mL}$ ($P < 0.05$).

Moreover, we examined whether MK615 was able to inhibit Aurora kinases in the pancreatic cancer cell lines. Aurora kinases mRNA expression is shown in Figure 3. The inhibition rate of Aurora A and Aurora B mRNA was $38.4\% \pm 2.9\%$ ($P = 0.02$) and $40.8\% \pm 3.2\%$ ($P = 0.03$) in PANC1 cells, $7.6\% \pm 1.1\%$ ($P = 0.05$) and $43.4\% \pm 4.5\%$ ($P = 0.03$) in PK1 cells, and $8.7\% \pm 2.5\%$ ($P = 0.05$) and $10.5\% \pm 2.1\%$ ($P = 0.05$) in PK45H cells, respectively, indicating a significant inhibition of mRNA expression of both Aurora kinases by MK615.

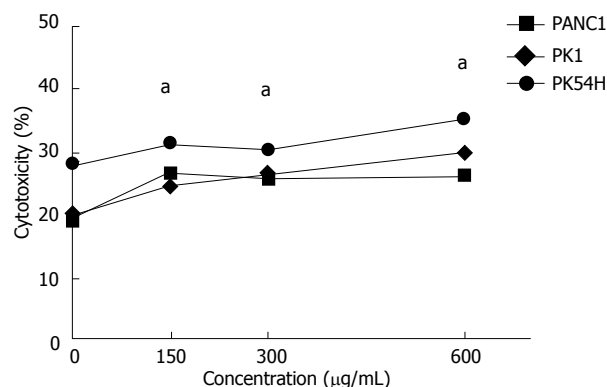


Figure 2 Lysis of pancreatic cells by MK615. MK615 lysed PANC1, PK1, and PK45H cells at concentrations of 150, 300, and 600 $\mu\text{g/mL}$. The percentage cytotoxicities at 150, 300, and 600 $\mu\text{g/mL}$ MK615 were significantly higher than those at 0 $\mu\text{g/mL}$ MK615 ($^*P < 0.05$). Cells were cultured for 48 h. The assay was carried out in triplicate.

Figure 4 shows the protein expression of Aurora kinases. After 48 h of incubation with 300 $\mu\text{g/mL}$ MK615, expressions of both Aurora A and B proteins were decreased in PANC1 and PK45H cells. Because endogenous Aurora A expression was low in PK1 cells, no significant decrease in its expression was observed. Consequently, MK615 decreased NF- κ B expression in PANC1 and PK45H cells, but no evident decrease in NF- κ B expression in PK1 cells was observed. The decrease in NF- κ B expression was more evident with the use of anti-phosphorylated NF- κ B antibody [pNF- κ B (ser536)].

In addition, we analyzed the effect of MK615 on the cell cycle. MK615 increased the proportion of cells in G2-M phase (Table 1) from 7.8% to 13.0% in PANC1 cells, from 1.8% to 5.2% in PK1 cells, and from 22.9% to 29.3% in PK45H cells.

DISCUSSION

MK615 is isolated from Japanese apricot and contains many defined and undefined substances, including several triterpenoids^[1], some of which exert an anti-neoplastic effect. In the present study, MK615 inhibited the proliferation of all three pancreatic cancer cell lines (Figure 1), and LDH assay showed that the anti-proliferative effect of MK615 on these cells was due not only to cell anti-proliferative effect, but also to direct cell killing effect (Figure 2).

In a previous study from our laboratory, MK615 was shown to be capable of inhibiting Aurora kinase A in human hepatocellular carcinoma^[3]. This prompted us to investigate the effects of MK615 on Aurora B kinases. Aurora kinases are key mediators of cell division by controlling chromatid segregation^[6-8]. There are three isoforms of Aurora kinases in mammals, Aurora A, Aurora B, and Aurora C. Structurally, all three share a similar domain organization, consisting of the N- and C-terminal domains, and a central region containing the catalytic domain, whose sequence is conserved among all three Aurora kinases^[9]. Despite their sequence similarities, however, the functions and cellular localization of Aurora kinases differ. Aurora A is localized at the centrosome and forms the mitotic spindle apparatus

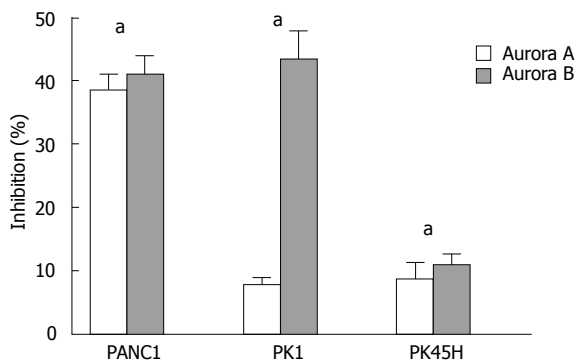


Figure 3 Dual inhibition of mRNA expression of Aurora A and Aurora B kinases by MK615. MK615 significantly inhibited the transcription of Aurora A and Aurora B mRNA. The percentage inhibition in PANC1, PK1, and PK45H cells was statistically significant ($P < 0.05$). RNA was extracted from the cells cultured with MK615 at a concentration of 300 $\mu\text{g/mL}$ for 48 h.

that plays a crucial role in segregating chromosomes into daughter cells. Aurora B is localized in inner-centromeric chromatin during metaphase-anaphase transition, and relocalizes to microtubules in the spindle midzone during telophase, being required for accurate cytokinesis^[10,11]. Unlike Aurora A and Aurora B kinases, the function of Aurora C still remains unclear. Over-expression of Aurora A and Aurora B is frequently observed in various human cancers^[12-15].

Our results demonstrated that MK615 inhibited the growth of, and showed cytotoxicity against, all three pancreatic cancer cell lines. The former effect was dose-dependent, while the latter was not. Although the mechanism responsible for the anti-neoplastic effect of MK615 has not been fully elucidated, the results of real-time PCR (Figure 3) and Western blotting (Figure 4) indicated that MK615 inhibited the expression of Aurora A and B kinases. There are several inhibitors of Aurora kinases, including the anti-Aurora B inhibitor, hesperadin^[16], anti-Aurora A and B, ZM447439^[17], and anti-Aurora A, B, and C, VX-680^[18]. As shown in the present study, MK615 dually inhibited both Aurora A and B kinases. Inhibition of Aurora kinases mRNA expression varied in three pancreatic cancer cell lines. In PANC1 and PK45H cells, Aurora A and Aurora B mRNA expression was inhibited to the same degree, whereas Aurora B mRNA was inhibited more efficiently by MK615 than Aurora A mRNA in PK1 cells. Consequently, MK615 efficiently inhibited Aurora A and Aurora B in PANC1 and PK45H cells at the protein level (Figure 4), whereas such inhibition was not evident in PK1 cells. Because the protein expression of Aurora A in PK1 cells was weak, the deficient inhibition of Aurora kinases may be due to the low expression of endogenous Aurora A in these cells. Although MK615 suppresses unknown molecules located upstream from Aurora A and B kinases, administration of MK615 at least down-regulates the expression of Aurora A and B kinases in a dual manner.

NF- κ B plays a central role in cell proliferation^[19-21] and is consistently activated in some human cancers^[22-24]. Aurora A binds the E2 ubiquitin ligase UBE2N, and UBE2N phosphorylates I κ B α . Activated I κ B translocates NF- κ B from the cytoplasm to the nucleus, resulting in

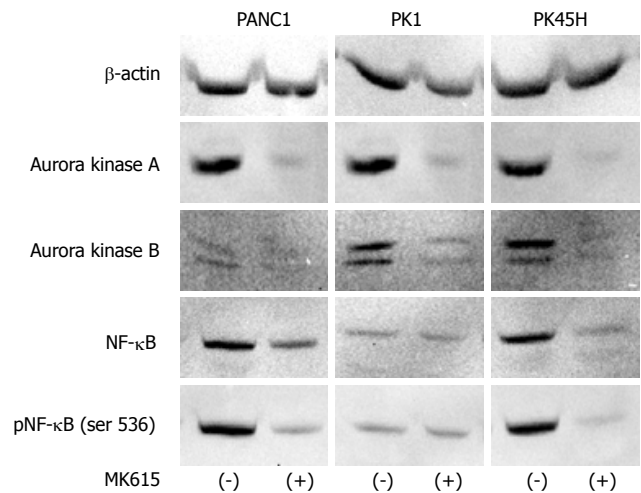


Figure 4 Inhibition of protein expression of Aurora A and Aurora B kinases, and NF- κ B by MK615. MK615 dually inhibited the expression of Aurora A and Aurora B kinases in PANC1 and PK45H cells. However, no inhibition was evident in PK1 cells. NF- κ B inhibition was more evident when anti-phosphorylated NF- κ B antibody was used. Protein was extracted from the cells cultured with MK615 at a concentration of 300 $\mu\text{g/mL}$ for 48 h.

activation of the NF- κ B complex^[25]. MK615 inhibits the expression of Aurora A, resulting in inhibition of NF- κ B activation^[26-28].

A previous study from our laboratory showed that MK615 induced cell-cycle arrest at the end of G2 phase in hepatocellular carcinoma^[3]. In the present study, MK615 treatment increased the cell population in G2-M phase in all three pancreatic cancer cell lines. MK615 inhibits Aurora A and Aurora B kinases and may induce cancer cell death at the G2-arrest check point^[29,30].

In conclusion, MK615 exerts an anti-neoplastic effect on human pancreatic cancer cells *in vitro* via dual inhibition of Aurora A and B kinases. Although further investigation is needed, MK615 seems to have the property anti-Aurora kinase compound.

COMMENTS

Background

MK615 is an extract from the Japanese apricot. Previous studies have revealed that MK615 has anti-neoplastic effects against human cancers. The mechanisms of anti-neoplastic effect of MK615 include apoptosis, autophagy, and suppression of Aurora A kinase. However, the entire mechanisms of the anti-neoplastic effects of MK615 have not been elucidated.

Research frontiers

Pancreatic cancer is one of the leading causes of death worldwide, and the 5-year survival rate is 2%-20%. There has been a need for chemotherapeutic drugs that are less toxic and can inhibit or suppress pancreatic cancer, and MK615 is a promising candidate.

Innovations and breakthroughs

There are not many inhibitors of Aurora kinases. MK615 is a natural compound which strongly inhibits Aurora A and B kinases.

Applications

MK615 shows an anti-neoplastic effect on human pancreatic cancer cells *in vitro* via dual inhibition of Aurora A and B kinases. MK615 may prove to be a therapeutic approach in pancreatic cancer therapy.

Terminology

MK615 is an extract from the Japanese apricot, *Prunus mume* Siebold et Zuccarini (ume in Japanese). Aurora kinases are key mediators of cell division by controlling chromatid segregation. There are three isoforms of Aurora kinases in mammals: Aurora A, Aurora B, and Aurora C.

Peer review

This is an *in vitro* study of an anti-neoplastic drug MK615 made from Japanese apricot. Its mechanism of action was studied and revealed to have inhibitory effects on Aurora A and B kinases. This is a good basic research on anti-neoplastic agent MK615 for pancreatic carcinoma.

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Management of cholelithiasis in Italian children: A national multicenter study

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development of complications was reported.

CONCLUSION: The therapeutic strategies were extremely heterogeneous. Ursodeoxycholic acid was ineffective in dissolution of gallstones but it had a positive effect on the symptoms. Laparoscopic cholecystectomy was confirmed to be an efficacy and safe treatment for pediatric gallstones.

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Key words: Pediatric cholelithiasis; Ursodeoxycholic acid; Laparoscopic cholecystectomy; Gallstones; Children

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Abstract

AIM: To evaluate the management of Italian children with cholelithiasis observed at Pediatric and Surgical Departments linked to Italian Society of Pediatric Gastroenterology Hepatology and Nutrition.

METHODS: One-hundred-eighty children (90 males, median age at diagnosis 7.3 years; range, 0-18 years) with echographic evidence of cholelithiasis were enrolled in the study; the data were collected by an anonymous questionnaire sent to participating centers.

RESULTS: One hundred seventeen patients were treated with ursodeoxycholic acid; in 8 children dissolution of gallstones was observed, but the cholelithiasis recurred in 3 of them. Sixty-five percent of symptomatic children treated became asymptomatic. Sixty-four patients were treated with cholecystectomy and in only 2 cases a postoperative complication was reported. Thirty-four children received no treatment and were followed with clinical and echographic controls; in no case the

INTRODUCTION

In the last years following the extensive use of ultrasound scanning an increasing number of children with cholelithiasis has been identified. In a population based study, prevalence of gallstones and biliary sludge in children was of 1.9% and 1.46%, respectively^[1]. In an analogue study, performed in Italian adults, the overall prevalence of gallstones diseases was of 9.5% in men and 18.9% in women^[2].

Gallstones disease is the most common and costly of all digestive disease in the United States, resulting in 700 000 cholecystectomies and as many as 1 000 000 hospitalizations annually^[3]. The frequency of hospital admission and operation for gallstones increased in Western countries since the 1950s^[4]. In England the age-standardized hospital admission rate for cholelithiasis enhanced from 68.7 to 104.9 per 100 000 population between 1989/1990 and 1999/2000 and it increased progressively with age from 1.1 per 100 000 in the 0-14 year age group to 277.1 per 100 000 in the ≥ 85-year age group in 1999/2000^[5].

Guidelines for management of cholelithiasis are available for adults. Cholecystectomy is recommended for

symptomatic patients and for those asymptomatic with a predisposition for malignancy (calcified gallbladder wall or family history of gallbladder cancer). For asymptomatic patients an expectant management with periodical clinical and echographic controls is recommended^[6,7]. On the contrary, little is known about natural history and management of cholelithiasis in childhood. So far, guidelines for management of children with gallstones are lacking. Surgical options for pediatric cholelithiasis include open or laparoscopic cholecystectomy^[8]. At the present, laparoscopic technique is considered the gold standard for cholecystectomy in children for reduced pain, absence of an upper abdominal incision and scar formation and a shorter period of hospitalization^[9-12]. The non-surgical approach is based on the use of ursodeoxycholic acid (UDCA); UDCA can reduce cholesterol saturation in bile and progressively dissolve cholesterol gallstones^[13,14]. Treatment of radiolucent gallstones with UDCA has been well documented in adults^[15-17]; on the contrary, little information is available for children^[18,19]. Another approach to pediatric cholelithiasis is represented by expectant management, in which the patients are not treated with medical or surgical therapies but are followed up with periodical clinical and ultrasound controls.

The aim of our multicenter retrospective study was to evaluate the management of Italian children with cholelithiasis observed at Pediatric and Surgical Departments linked to Italian Society of Pediatric Gastroenterology Hepatology and Nutrition (SIGENP).

MATERIALS AND METHODS

Subjects

A retrospective study aimed to evaluate the management of the Italian children with cholelithiasis observed at Medical and Surgical Pediatric Departments linked to SIGENP during the period 1995-2005 was performed. An anonymous questionnaire that investigates age, sex, clinical presentation of cholelithiasis, risk factors for cholelithiasis, basal liver function tests and ultrasonographic findings was proposed to SIGENP members. Seven departments (six medical and one surgical) agreed to participate to the study, three in Northern Italy, one in central regions and three in southern Italy. Each department sent data to the Coordinating Centre in Naples for statistical analysis. The SIGENP approved the project. All patients with echographic evidence of cholelithiasis with age range 0-18 years were enrolled in the present study. The diagnosis of cholelithiasis was based on the presence of echogenic foci that produced acoustic shadowing in the gallbladder or in the region of gallbladder fossa. Sludge was defined as non-shadowing, echogenic, intraluminal sediment. When the sludge was seen at the same time of gallstones, the children were considered to have cholelithiasis. The children with diagnosis of biliary sludge were excluded from the present study. Gallstones were distinguished in radio-lucent and radio-opaque on the basis of radiographic aspect. In all patients risk factors for cholelithiasis such as hemolytic disorders, familiarity for gallstones, obesity, total parenteral nutrition (NPT), hepatobiliary chronic disease, cystic fibrosis, therapy with ceftriaxone, abdominal surgery,

IgA deficiency and Gilbert's disease were investigated. The gallstones in patients without risk factors for cholelithiasis were considered idiopathic. With regard to clinical presentation the patients were distinguished in two groups: asymptomatic and symptomatic. Asymptomatic children had no abdominal discomfort or gastrointestinal complaints; their gallstones were diagnosed incidentally on ultrasound examinations for causes unrelated to cholelithiasis. As previously reported, symptomatic children were divided into those with colicky pain, those with typical biliary tract symptoms (right upper quadrant or epigastric pain, nausea, vomiting and food intolerance) and with atypical symptoms^[1].

Management

For each patient, type of treatment and outcome were evaluated. For patients treated with UDCA, therapy was considered effective in case of complete gallstone dissolution at ultrasound scanning and disappearance of clinical symptoms. For surgically treated patients, efficacy of treatment was evaluated on the basis of disappearance of clinical symptoms without the development of "post-cholecystectomy syndrome". Gallstone recurrence was defined as detection of echogenic foci in the gallbladder that produced acoustic shadowing, after that at least in an occasion disappearance of gallstones was documented at ultrasound scanning. We recorded clinical, laboratory and echographic features of all patients, type of treatment and outcome.

Statistical analysis

Data were analyzed with the χ^2 test and with Fisher's exact test and Student's *t*-test as appropriate. *P* < 0.05 was considered significant.

RESULTS

During the period 1995-2005, 196 children with gallstones or biliary sludge (98 males; median age at diagnosis 7.3 years; range, 0-18 years) were observed at Pediatric Medical Departments of Naples (56 cases), Milan (22 cases), Rome (19 cases), Bari (16 cases), S. Giovanni Rotondo (7 cases), Ferrara (6 cases) and at Pediatric Surgical Department of Brescia (70 cases). 181 patients (92.3%) had gallstones and 15 (7.7%) had biliary sludge and so were excluded from the study. In the patients with gallstones, the sex ratio was equal, but in the adolescent group (12-18 years) there was a female predilection for gallstones. Eighty-six (47.5%) children showed one or more risk factors for gallstones at personal and familial anamnesis (Table 1). Ninety-five (52.5%) patients had no risk factors and their cholelithiasis was defined as idiopathic. Presence of one or more relatives with cholelithiasis represented the most common risk factor at any age, with a sensitive increase of frequency with age (Figure 1). Positive family history for cholelithiasis was described in maternal branch in 27 patients (52.9%), in the paternal in 12 cases (23.6%) and in both branches in 10 patients (19.6%), while in 2 (3.9%) children cholelithiasis was found in the brother and in the sister, respectively. Presence of relatives with cholelithiasis, obesity and hemolytic disorders were the predominant risk

Table 1 Risk factors for cholelithiasis in 181 children observed at Pediatric Medical and Surgical Department

Risk factor	Pediatric medical departments	Pediatric surgical department	Total
Hemolytic disorders	9	7	16
Familiarity	46	5	51
Obesity	14	0	14
NPT ¹	0	4	4
Hepatobiliary chronic disease	8	1	9
Cystic fibrosis	2	0	2
Ceftriaxone therapy	4	7	11
Abdominal surgery	2	0	2
IgA deficiency	1	0	1
Gilbert's disease	2	1	3
No risk factors identified	55	40	95

¹NPT: Total parenteral nutrition.**Table 2** Clinical characteristics, type of treatment and outcome in 119 children with cholelithiasis observed at Pediatric Medical Department

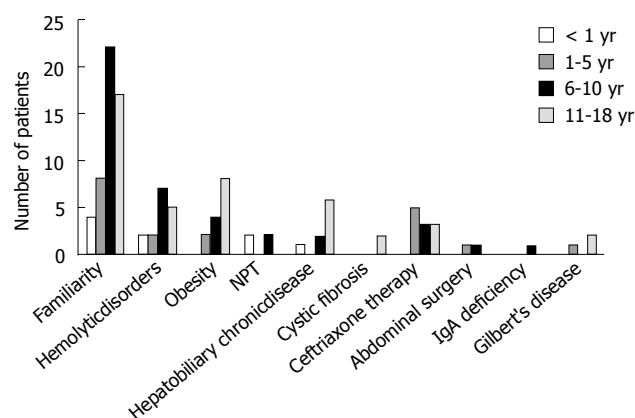
	UDCA therapy	Cholecystectomy	Wait and see
Asymptomatic	33	2	17
Colicky pain	18	7	6
Typical biliary tract symptoms	26	4	7
Atypical symptoms	6	0	3
Pancreatitis/Cholecystitis	0	1	0
Resolution of lithiasis	6	14	0
Recurrence of lithiasis	3	0	0
Resolution of symptoms	40	14	0
Recurrence of symptoms	0	3	0
Total of patients	83	14	33

Table 3 Clinical characteristics, type of treatment and outcome in 62 children with cholelithiasis observed at Pediatric Surgical Department

	UDCA therapy	Cholecystectomy	Wait and see
Asymptomatic	8	9	1
Colicky pain	12	23	0
Typical biliary tract symptoms	9	14	0
Atypical symptoms	3	3	0
Pancreatitis/Cholecystitis	2	1	0
Resolution of lithiasis	2	50	0
Recurrence of lithiasis	0	0	0
Resolution of symptoms	9	50	0
Recurrence of symptoms	0	0	0

factors in children with gallstones. According to presence of symptoms at diagnosis, the patients were divided in asymptomatic (64 patients, 35.3%) and symptomatic (117 patients, 64.7%).

In the context of the symptomatic children with regard to clinical presentation, four groups of patients could be distinguished: 51 (43.6%) patients (28 males, median age 10 years; range, 3 mo-18 years) with colicky pain with or without jaundice; 50 (42.7%) patients (25 males, median age 7 years; range, 2 mo-16 years) with typical chronic

**Figure 1** Risk factors for cholelithiasis and age at diagnosis.

biliary tract symptoms; 12 patients (10.3%) (3 males, median age 6.5 years; range, 3 mo-14 years) with atypical symptoms; 4 (3.4%) children (2 males, median age 5.6 years; range, 2-13 years) with pancreatitis/cholecystitis.

The distribution of clinical presentations in the different age groups showed that the rate of colicky pain was higher in older children; instead as for the other clinical presentations, there was no correlation with age at diagnosis. According to therapy, patients could be divided in 3 groups: 117 (64.6%) children treated with UDCA, 64 (35.3%) children treated with laparoscopic or laparotomic cholecystectomy and 34 (18.8%) observed with expectant management. It is to note that 34 children were treated with UDCA in a first phase and thereafter, for persistence of gallstones, with cholecystectomy.

The clinical characteristics, type of therapy and outcome of studied patients are reported in Tables 2 and 3.

Patients observed at medical departments

Medical treatment: Eighty-three (69.7%) of 119 patients with gallstones observed at Medical Departments were treated with UDCA (25 mg/kg per day; range 18-30 mg/kg per day) for median period of 13 (range, 3-96) mo. Thirty-three (39.8%) patients had radiolucent gallstones, 17 (20.4%) radio-opaque gallstones, while 33 patients (39.8%) did not undergo radiographic examination. The median of diameter maximum of gallstones was 8 (range, 2-45) mm. All children completed the therapy without any adverse effects. During UDCA therapy they underwent an ultrasound scanning every 3-6 mo. The gallstones completely disappeared in only 6 (7.2%) children (4 radio-lucent and 2 with unknown radiographic-aspect), but the cholelithiasis recurred in 3 (50%) of them; in one child, gallstones reappeared after 6 years and in the other two biliary sludge were observed after 6 and 10 mo, respectively. Four of 6 patients with gallstones dissolution were symptomatic at diagnosis; all 4 children were relieved of their symptoms during UDCA therapy. The other 2 children remained asymptomatic during the observation period. In other 4 patients disappearance of gallstones was observed, but three of them had a pseudolithiasis (following antibiotic-therapy with ceftriaxone) and one had fetal gallstones. Since these conditions are described as auto-resolving diseases^[20-23], the disappearance was not

considered induced by UDCA. In 73 (87.9%) patients treated with UDCA there was persistence of gallstones. Forty-three patients were symptomatic at diagnosis. In 36 (83.7%) of these patients resolution of clinical discomfort was observed, while in 7 (16.3%) of them symptoms persisted unchanged during the therapy. The other 30 (41.1%) children remained asymptomatic during the treatment. Eleven (13.2%) patients underwent cholecystectomy at the suspension of medical therapy for either recurrence or persistence of symptoms. Significant differences in terms of sex, age, characteristics of gallstones (diameters, Rx-aspect) and risk factors for cholelithiasis were not found among the patient's responders and non-responders to UDCA.

Surgical treatment: Fourteen (11.8%) patients with gallstones observed at Pediatric Medical Departments were surgically treated. At x-ray examination, 4 (28.6%) of them had radiolucent gallstones, 6 (42.8%) radio-opaque stones; in the remaining 4 (28.6%) cases the radiographic aspect of gallstones was unknown. Eleven (78.6%) patients were symptomatic and 3 (21.4%) asymptomatic. The median of diameter maximum of gallstones was 7 (range, 3-33) mm. In 10 (71.4%) cases cholecystectomy was performed by laparoscopic techniques and in 4 (28.6%) by laparotomy. There were no post-operative complication and in no case residual cholelithiasis or recurrence of gallstones during the follow-up were observed. In three patients recurrence of clinical symptoms after treatment (post-cholecystectomy syndrome) was described.

Expectant management: Thirty-three (27.7%) patients were not treated, but they were observed with clinical, laboratory and ultrasonographic controls every 3-6 mo. Two patients (6%) had radiolucent stones, 16 (48.5%) radio-opaque cholelithiasis and the other 15 (45.5%) had not been submitted to x-ray examination. The median of diameter maximum of gallstones was 9 (range, 2-23) mm. In no case development of complication was observed and none of the 17 (51.5%) asymptomatic patients became symptomatic during the follow up (median duration of follow-up, 9 mo, range 1-45).

Patients observed at surgical department

Seventy patients enrolled in the present study come from the Pediatric Surgical Department of Brescia. To avoid selection bias in the evaluation of the treatment and the outcome we have decided to separately analyze the children of this center.

Sixty-two patients had gallstones and 8 had biliary sludge and so were excluded from the study (median age 7 years; range, 0-18 years). Forty-eight (77.4%) patients were symptomatic at diagnosis, while 14 (22.6%) were asymptomatic. Thirty-four children (54.8%) with gallstones (21 radio-lucent, 9 radio-opaque, and 4 unknown radiographic aspect) were treated with UDCA (median dose 18 mg/kg per day; range, 5-30 mg/kg per day; median duration 5 mo; range, 2-36 mo). Twenty-six patients were symptomatic and eight were asymptomatic. Following UDCA therapy, nine (34.6%) of 26 symptomatic patients became symptom free, while in 17 (65.4%) cases

the symptoms persisted unchanged during the therapy. Gallstones completely disappearance in 2 (5.9%) radiolucent cases. In other 2 patients spontaneous resolution of lithiasis was observed (pseudo-lithiasis induced by ceftriaxone). In the remaining thirty (88.2%) patients (17 radiolucent gallstones, 9 radio-opaque and 4 with unidentified radiographic-aspect) UDCA was inefficacy with persistence of gallstones despite therapy. Twenty-three of these children underwent cholecystectomy, while 7 were followed with periodical controls. The other 27 patients were directly treated with surgical approach. In forty patients (80%) cholecystectomy was performed by laparoscopic technique and in 10 (20%) cases by laparotomic approach. Histological analysis of the removed gallbladders was available for 39 patients: thirty-three (84.6%) had chronic cholecystitis, 2 (5.1%) papillomatosis, 1 (2.6%) adenoma of gallbladder and only 3 (7.7%) patients had normal gallbladder wall. Postoperative complications were not reported except in a child who developed a laparocele and in another who had dilatation of choledochus duct. In no case the post-cholecystectomy syndrome was observed.

Only one (1.6%) patient was not treated and followed with periodical clinical and echographic controls.

DISCUSSION

Although cholelithiasis is considered as an uncommon condition in children, recent series document an increasing detection of this disorder^[1]. This increase may be explained by the increased use of abdominal ultrasound scanning in childhood. The frequency of pediatric gallstones, documented in studies in which patients were selected according to initial symptoms, resulted in prevalence's between 0.13% and 0.22%^[1,24]. In Wesdorp's study, in which patients with cholelithiasis were obtained by screening 4200 abdominal ultrasound scans performed for different reasons such as typical biliary symptoms, general abdominal symptoms or routinely check-ups, a higher prevalence of gallstones and biliary sludge (1.9% and 1.46%, respectively) was observed. For its typology the present study cannot give information on the epidemiology of pediatric cholelithiasis, but it has however confirmed some aspects previously reported, as major frequency in the female only after puberty, increase of the cases with age, association with some risk factors such as familiarity for cholelithiasis, obesity and hemolytic disorders^[1,25,26]. It was previously reported that approximately 80% of adults with gallstones are asymptomatic^[2,27]. Instead we have found that only one third of children with cholelithiasis were asymptomatic. This could be explained by the tertiary nature of involved centers or may indicate that asymptomatic cholelithiasis is less frequently in children, as described in other studies^[1].

Although the prevalence of cholelithiasis in children is increasing, little information is available about the management of this disorder in childhood. There are only few data on pediatric cholelithiasis and in the majority of cases the studies were performed on a limited number of patients. In our study, in which the management of cholelithiasis was evaluated in a large cohort of children observed at Medical and Surgical Department linked

to SIGENP, both diagnostic approach and therapeutic strategies were extremely heterogeneous. As for diagnostic approach, abdominal X-ray was not performed in all cases; in fact, radiographic aspect of gallstones in a third of patients was not evaluated. As for treatment, although in adults UDCA therapy is recommended only for a specific subset of patients^[15], in the present study this drug was used in about two third of cases and only in half of cases the gallstones were radiolucent. Our study confirmed that UDCA was ineffective in dissolution of gallstones in the vast majority of the cases. In addition, there was an important rate of recurrence of cholelithiasis after primary dissolution. Instead UDCA had a positive effect on the symptoms determining the disappearance of abdominal discomfort in the vast majority of the symptomatic patients treated.

In our study laparoscopic cholecystectomy was confirmed to be an efficacy and safe procedure in the treatment of pediatric gallstones for the low rate of post-operative complications (3%) and post-cholecystectomy syndrome (4.7%). In contrast to Wesdorp's study, in which 45% of children with biliary symptoms or colicky pain had recurrence of their symptoms after treatment (Endoscopic Retrograde Cholangiopancreatography (ERCP) or cholecystectomy), in our study the post-cholecystectomy syndrome was observed only in a small percentage of cases. This difference may be explained by the greater recurrence of symptoms associated with ERCP than with cholecystectomy.

The histological analysis of the removed gallbladders showed structural alterations such as chronic cholecystitis, papillomatosis and adenoma in the majority of cases; in only few cases the gallbladder wall was normal. Therefore, for long life expectancy of children, expectant management may not be safe in pediatric patients. In fact, in contrast to adults in whom the natural history of gallstones is well documented, in children it is not known. In adults only 1%-4% per year will develop symptoms or complications of gallstones disease; only 10% will develop symptoms in the first five years after diagnosis and approximately 20% by 20 years^[28-30].

A fifth of patients with gallstones enrolled in our study were not treated and followed with periodical clinical and echographic controls; in none of them any complication occurred during the follow-up (median 9 mo; range, 3-45). It is to note, however, that in our study the observation period of untreated was too short to evaluate the effective rate of complications.

Considering the typology of the present study and the heterogeneous management observed in our patients, we are unable to provide clear indications for management of cholelithiasis in children. However on the basis of the available studies and our results, the following approach might be suggested. Firstly, the children with gallstones should be divided in two groups on the basis of clinical presentation: symptomatic and asymptomatic. As for asymptomatic gallstones, considering the low rate of complications observed and the favorable natural history described in adults, we recommend an expectant management with periodical clinical and ultrasonographic

controls. On the other hand, as for symptomatic gallstones, considering the low rate of post-operative complications and post-cholecystectomy syndrome, we suggest surgical approach with laparoscopic cholecystectomy. For its inefficacy on dissolution of gallstones and for high recurrence rate of gallstones, UDCA should not be used in pediatric gallstones, except in symptomatic children with contraindication to surgery for reduce the clinical symptoms.

It is necessary that this approach is validated in further studies including a larger number of children; furthermore, it would be desirable to known in larger series the natural history of cholelithiasis in childhood.

COMMENTS

Background

Although recent extensive use of abdominal ultrasound scanning has identified an increased number of cholelithiasis, little is known about the natural history and management of this disorder in childhood.

Innovations and breakthroughs

Only few data on pediatric cholelithiasis are available and in the majority of cases the studies were performed on limited number of patients. In our study, the management of cholelithiasis was retrospectively evaluated in a large cohort of children observed at Medical and Surgical Department linked to Italian Society of Pediatric Gastroenterology Hepatology and Nutrition (SIGENP).

Applications

The present study suggests a possible approach to pediatric cholelithiasis. It is desirable, however, that this approach is validated in further prospective studies including a larger number of children.

Peer review

This retrospective study is well done and nicely written and focused on a relevant topic (cholelithiasis in children).

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Liver histology in ICU patients dying from sepsis: A clinico-pathological study

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the main histological findings in the liver in the majority of patients dying from sepsis.

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Abstract

AIM: To determine end-stage pathologic changes in the liver of septic patients dying in the intensive care unit.

METHODS: Needle liver biopsies obtained immediately after death from 15 consecutive patients with sepsis and no underlying liver disease were subjected to routine histological examination. Liver function tests and clinical monitoring measurements were also recorded.

RESULTS: Liver biochemistries were increased in the majority of patients before death. Histology of liver biopsy specimens showed portal inflammation in 73.3%, centrilobular necrosis in 80%, lobular inflammation in 66.7%, hepatocellular apoptosis in 66.6% and cholangitis/cholangiolitis in 20% of patients. Mixed hepatitic/cholestatic type of liver injury was observed in 6/15 (40%) patients and hepatitic in 9/15 (60%). Steatosis was observed in 11/15 (73.3%) patients affecting 5%-80% of liver parenchyma. Among the histological features, the presence of portal inflammation in liver biopsy was associated with increased hospitalization in the ICU prior death ($P = 0.026$).

CONCLUSION: Features of hepatitis and steatosis are

INTRODUCTION

Sepsis is the leading cause of death in patients admitted in intensive care units (ICUs)^[1]. Hepatic dysfunction represents a common manifestation during the sepsis process, ranging from a mild elevation of serum bilirubin and/or liver enzymes to severe hepatic failure^[2]. The pathophysiology of liver injury in sepsis is multifactorial and involves infection, drugs, metabolic disturbances and a broad spectrum of inflammatory mediators^[3,4]. The detailed histopathological findings in the liver of patients at the late stages of sepsis remain relatively unknown as scarce data are available in humans.

In the present study, we aimed to assess the pathologic changes in liver core-needle biopsies obtained immediately after death from ICU septic patients and to correlate these with clinical and laboratory data.

MATERIALS AND METHODS

Subjects

We evaluated liver core-needle biopsies, obtained immediately (within 5 min) after death, from 15 patients who were admitted because of sepsis in the ICU of the Hippocraton General Hospital of Athens. The modified sepsis criteria described by Bone *et al*^[5] were applied, including clinical suspicion of sepsis, hyper- or hypothermia

Table 1 Clinical data of patients admitted to ICU with sepsis

No	Age	Sex	Diagnosis	Cause of hospitalization	APACHE II	Cultured sample	Infective agent
1	58	Male	Respiratory infection	ARD	17	Blood	Klebsiella
2	70	Male	Respiratory infection	ARD	15	Blood	Klebsiella
3	84	Male	Respiratory infection	Heart failure	17	Respiratory (BAL)	Enterobacter
4	57	Male	Respiratory infection	AMI	23	Venous catheter	Klebsiella
5	50	Male	Soft tissue infection	Soft tissue infection	20	Skin	Staphylococcus epidermidis
6	74	Male	Intra-abdominal sepsis	Peritonitis	29	Blood	Enterobacter
7	45	Female	Intra-abdominal sepsis	Peritonitis	21	Venous catheter	Klebsiella
8	74	Female	Peritonitis	Peritonitis	30	Midstream urine sample	Candida albicans
9	86	Male	Sepsis	Sepsis	37	Venous catheter	Pseudomonas
10	75	Male	Respiratory infection	ARD	27	Blood	Pseudomonas
11	33	Male	Soft tissue infection	Burn injury	7	Blood	Acinetobacter
12	69	Male	Peritonitis	Peritonitis	17	Blood	Klebsiella
13	72	Female	Respiratory infection	ARD	15	Blood	Klebsiella
14	70	Male	Respiratory infection	AMI	26	Venous catheter	Klebsiella
15	77	Male	Respiratory infection	Aneurysm	20	Respiratory (BAL)	Candida

ARD: Acute respiratory distress; AMI: Acute myocardial infarction; BAL: Bronchoalveolar lavage.

(rectal temperature $> 38.5^{\circ}\text{C}$ or $< 35^{\circ}\text{C}$), tachycardia, and at least one of the following indications of altered organ perfusion and function: altered mental status, hypoxemia, elevated serum lactate level and oliguria. Exclusion criteria included pre-existing hepatobiliary disease, HIV infection and malignancies. In all patients, the standard treatment for the managing of sepsis was used according to the Surviving Sepsis Campaign guidelines^[6]. Death was attributed to multiple organ failure due to sepsis and was defined as the loss of cardiac rhythm which could not be recovered after initial resuscitation. Support was withdrawn from patients with spontaneous cardiopulmonary arrest.

Methods

Informed consent was obtained from the patients' relatives in all cases. All liver biopsies were performed by the same ICU consultant physician within first 5 min from resuscitation refractory cardiac arrest as defined by three cycles of defibrillator shock. Biopsy specimens were fixed in 10% neutral buffered formalin and embedded in paraffin.

Causative infective agents, sites of infection, causes of admission in the ICU and/or transfer from another department, initial APACHE II Score as well as patients' demographics are shown in Table 1. Initial laboratory values were measured upon admission to ICU, while the most recent values obtained before the reported time of death, were considered as terminal laboratory values.

Histopathology

Liver biopsies measured 17-46 (mean 25) mm in length and contained 6-28 (mean 12) portal tracts. Serial sections of liver biopsies were cut at 4- μm intervals and mounted on slides coated with poly-L-lysine. Sections were stained with hematoxylin and eosin and with special histochemical stains (reticulin, Masson Trichrome, PAS, PAS-diastase, Prussian blue and Gram) for detailed morphological evaluation by two independent pathologists. Type, zonal location and amount of steatosis were recorded for each case. The latter was graded as 0 ($< 5\%$ of parenchymal

involvement), 1 (5%-33%), 2 ($> 33\%$ -66%) and 3 ($> 66\%$), according to previously published criteria^[7]. Apoptotic (acidophilic) bodies were evaluated as the total count in the liver specimen and as the number per mm^2 of examined liver tissue.

Statistical analysis

Data were analyzed using SPSS 13.0 software. All continuous variables are reported as the median and range. Relationships between categorical variables were tested with χ^2 analysis, with Fisher's exact test were applicable. Quantitative variables were analysed using non-parametric statistics (Mann-Whitney *U* test for two independent samples). $P < 0.05$ were considered statistically significant.

RESULTS

Clinical and laboratory data

Patients' clinical parameters and laboratory data at entry and before death are shown in Table 2. At entry, 2 patients (13.3%) presented with elevated bilirubin levels, while increased AST was present in 7/15 (46.7%), abnormal ALT in 8/15 (53.3%), γGT in 6/15 (40%) and ALP in 2/15 (13.3%) patients. Before death, elevated serum levels of bilirubin, AST, ALT, γGT and ALP were observed in 8/15 (53.3%), 8/15 (53.3%), 7/15 (46.7%), 14/15 (93.3%) and 11/15 (73.3%) patients, respectively. In summary, an overall deterioration of liver-related biochemical parameters was observed in all our patients. With respect to the type of liver injury, 3/15 (20%) patients showed a clearly cholestatic biochemical profile, with substantial elevation of γGT and ALP and normal or nearly normal AST and ALT levels (< 1.5 the upper normal limit), while 12/15 (80%) patients exhibited a mixed hepatic and cholestatic profile.

Histology

The observed histological findings in liver biopsy specimens were portal inflammation in 11/15 (73.3%, mixed in 8 patients and lymphohistiocytic in 3), centrilobular necrosis in 12/15 (80%), lobular inflammation in 10/15 (66.7%),

Table 2 Clinical and laboratory parameters in patients who died from sepsis at entry and before death (Data are presented as median, range)

Variable	Initial	Final	P-value
Heart rate (/min)	88 (50-150)	86 (76-140)	NS
Systolic blood pressure (mmHg)	130 (70-180)	110 (60-150)	0.017
Diastolic blood pressure (mmHg)	70 (45-80)	50 (40-80)	0.018
PO ₂ (mmHg)	154 (50-579)	81 (45-120.8)	0.008
PCO ₂ (mmHg)	42 (23-138)	38 (28.1-81)	NS
pH	7.36 (7.14-7.46)	7.33 (6.93-7.46)	NS
HCO ₃ (mmol/L)	24 (11.3-31.2)	18.5 (11-32.4)	NS
Bilirubin (μmol/L)	17.1 (6.84-107.73)	23.94 (8.55-356.13)	NS
AST (nKat/L)	600 (230-2230)	650 (150-4160)	NS
ALT (nKat/L)	680 (150-4170)	450 (100-2730)	NS
ALP (nKat/L)	1080 (45-3550)	2250 (930-4630)	0.019
γGT (nKat/L)	850 (180-6700)	2030 (630-5720)	NS

NS: Non-significant.

Table 3 Clinical and laboratory parameters according to morphologically determined apoptosis in liver

Parameter	Liver apoptosis		P-value
	Yes (n = 10)	No (n = 5)	
APACHE II	17 (7-26)	18.5 (15-37)	NS
Heart rate (min)	86 (76-124)	93 (76-140)	NS
Systolic blood pressure (mmHg)	105 (60-150)	112.5 (85-145)	NS
Diastolic blood pressure (mmHg)	50 (40-80)	55 (50-65)	NS
PO ₂ (mmHg)	89.5 (49-120.8)	77.5 (45-110)	NS
pH	7.34 (7.09-7.46)	7.29 (6.93-7.38)	NS
Bilirubin (mmol/L)	23.94 (11.97-356.2)	42.7 (8.55-140.22)	NS
Direct bilirubin (mmol/L)	17.442 (5.13-230.166)	53.1 (10.26-136.166)	NS
AST (nKat/L)	683.47 (433.42-3717)	516.8 (216.7-467)	NS
ALT (nKat/L)	733.48 (100-2733.9)	433.4 (250.1-1150.2)	NS
γGT (nKat/L)	2283.8 (1083.6-5717)	1467 (633.5-4500)	NS
ALP (nKat/L)	2300.5 (1700.3-4634.3)	1967.1 (933.2-3133)	NS

hepatocellular apoptosis in 10/15 (66.6%), cholangitis in 1/15 (6.6%), cholangiolitis in 2/15 (13.3%) patients, canalicular cholestasis in 1/15 (6.6%) and ductular cholestasis in 2/15 (13.3%) patients (Figure 1A-C). Damage to bile duct epithelium was not observed.

The type of liver injury was defined as mixed or hepatitic according to overall histological features; 9/15 (60%) patients had the “hepatitic” type when only portal/lobular inflammation and/or centrilobular, frequently hemorrhagic, necrosis were present (Figure 1A), while 6/15 patients (40%) had “mixed” histological liver injury characterized by different combinations of biliary lesions (bile duct and/or ductular hyperplasia, cholangitis, cholangiolitis), cholestasis (canalicular and/or ductular) (Figure 1B and C), portal/lobular inflammation and centrilobular necrosis. Gram stains for bacteria and PAS for fungi were negative in all cases.

Steatosis was observed in 11/15 (73.3%) patients, of whom 5 had grade 1 (parenchymal involvement range: 5%-20%), 3 grade 2 (40%-50%) and 3 grade 3 (70%-80%).

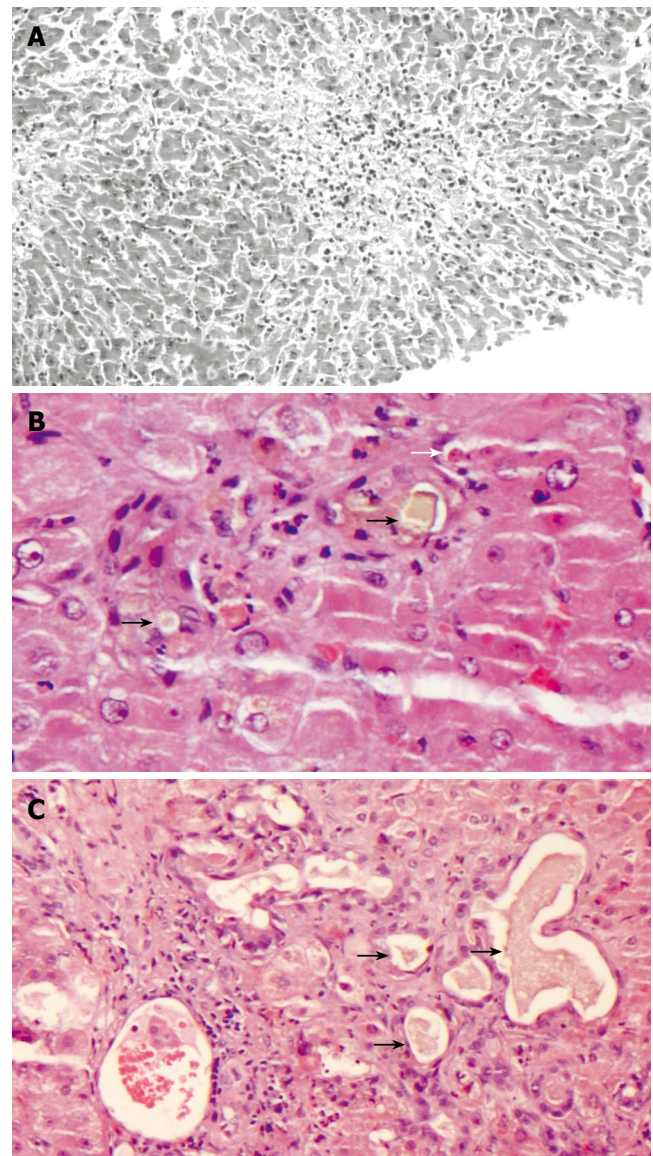


Figure 1 Histological findings in liver biopsy specimens. **A:** Zone 3 (centrilobular) necrosis (HE, x 100); **B:** Canalicular cholestasis. Bile plugs in dilated canaliculi (black arrows). An apoptotic (acidophilic) body is present (white arrow) (HE, x 200); **C:** Ductular cholestasis and inflammation. Dilated bile ductules at the margin of an inflamed portal tract are filled with bile (black arrows) (HE, x 100).

Four out of 11 patients had macrovesicular, 5/11 microvesicular and 2/11 mixed type of steatosis. Seven of 8 patients (87.5%) who developed hyperbilirubinemia before death had a mixed type of liver injury and one (12.5%) had a hepatitis-like liver injury, whereas those who did not have jaundice ($n = 7$) had no histological features of cholangitis/cholangiolitis. Apoptotic bodies, observed in 10/15 (66.6%) patients, were mainly seen in those who had lobular inflammation. The association of apoptosis with clinical and biochemical analysis at the closed time of death is seen in Table 3.

In order to examine whether the histological findings were associated to the duration of ICU hospitalization we performed univariate regression analysis. Patients with portal inflammation in biopsy specimens had a significant long term ICU hospitalization compared to patients without evidence of portal inflammation [no portal

inflammation *vs* portal inflammation: 3 (3-49) *vs* 25 (4-92) days Mann-Whitney *U* test, $P = 0.026$]. None of the other histological findings on liver found to be significantly associated with the length of ICU hospitalization.

DISCUSSION

In our study, we have observed that liver involvement in sepsis is common and characterized by either a hepatitic-like injury, observed in 60% of our patients, or a mixed, hepatitic and cholestatic, pattern of injury. Furthermore, steatosis was a common finding observed in 74% of our patients. Bacteraemia and sepsis have been associated with abnormal liver biochemistry^[2-4,8-10]. In the current study, elevated serum ALP and γ GT levels were observed in 70% and 93% of our cases before death compared to 13% and 40% at admission, respectively. Similarly, increased bilirubin levels were seen in 53% of our cases before death compared to 13% at admission. In this respect, serum bilirubin, γ GT and ALP may serve as indicators of clinical deterioration in septic critically ill patients. Other studies, however, have showed conflicting data regarding the prevalence of abnormal liver tests in patients with bacteraemia^[2,8-10]. These discrepancies can be attributed to differences in patients' selection criteria as well as in the severity of the underlying disease.

Liver histology in sepsis has been evaluated mainly in animal studies and in postmortem human liver tissue where a significant time period had elapsed between autopsy and time of death^[4,9,11-13]. In jaundiced septic patients, three histological patterns have been described in a limited number of studies^[4,9,11]: canalicular cholestasis, usually most severe in zone 3, ductular cholestasis with inflammation and non-bacterial cholangitis associated with the toxic shock syndrome^[12]. Intrahepatic cholestasis in septicemia could be attributed to many factors such as circulating endotoxin causing functional disorders in bile secretion, disturbances in bile canalicular contraction and ischemia^[14-17].

The lack of detailed histological data has led clinicians to evaluate sepsis-related liver damage from the serum biochemical markers while no studies have addressed the correlation between laboratory values and pathologic findings. In the current study, we have obtained liver tissue from our septic patients immediately after death leading to the most accurate identification of sepsis-related liver pathology. Our findings showed that sepsis is characterized by a "hepatitic" like liver injury in 60% of our patients and a "mixed", cholestatic and hepatitic type in 40% of them. Cholangitis and/or cholangiolitis were observed in a few patients. Additionally, ductular cholestasis, a sepsis-specific hepatic lesion^[11,12], suggesting increased risk of mortality^[16] was identified only in two of our patients dying of sepsis, while canalicular cholestasis was present in one. Damage to bile duct epithelium has been previously observed in the liver of a female patient with *E. coli* septicemia^[13]; however, it was not detected in any of our patients. The contrast between our histological findings and that of previous morphological studies where canalicular or ductular cholestasis predominated in the liver of jaundiced patients who died of sepsis, maybe attributed to the small

number of cases examined. The presence of mixed, cholestatic and hepatitic, features in the majority of our patients with hyperbilirubinemia may alternatively be the result of the direct effect of endotoxin or a drug-induced injury. Centrilobular hemorrhagic necrosis, which was common in our cases, is a frequent finding in livers from patients with peripheral circulatory failure^[19]. Among the above histopathological findings, the presence of portal inflammation, a common finding in chronic hepatitis, was associated with prolonged hospitalization that may reflect the effect of prolonged exposure to treatment medication.

Steatosis was evident in the liver of most of our septic patients. In previous studies, although steatosis was a common finding in the post-mortem liver of septic patients, its extent and type has not been examined in detail or commented on^[4,9,11]. The majority of our patients had moderate to severe fatty liver change comprising 40-80% of the liver parenchyma. It is known that sepsis and bacterial toxins may cause macrovesicular^[20] or microvesicular steatosis^[21] and hypoxia may play a role in these cases. Also, a wide variety of drugs and total parenteral nutrition may be responsible for the development of fatty liver change^[20].

Liver apoptosis can be ascribed to a wide variety of individually or simultaneously acting underlying mechanisms. Tissue hypoxia, inflammatory mediators, free radicals, bacterial toxins and drug toxicity are all implicated in the above mechanisms^[3,20,22-23]. The presence of apoptosis was much more common in patients with more severe liver histology, as defined by the intensity of portal and lobular inflammation as well as the presence of lobular necrosis and the ductular cholestatic lesions. Prognostic determinants cannot be inferred from the current study, since all our patients succumbed due to multiple organ failure. However, the absence of pre-existing liver disease, as well as the fact that all biopsies were optimally performed immediately after death, suggests that the subsequent pathologic evaluation demonstrates terminal sepsis-related histologic changes in the liver parenchyma.

Summarizing our results, the liver of end stage patients mainly shows histopathological features of hepatitis injury, with additionally cholestatic findings along with steatosis. Biopsies were performed almost immediately after each patients death giving the most precise evaluation of sepsis-induced liver injury. Further studies are needed to clarify the role of apoptosis and application of innovative drugs in sepsis-induced liver injury.

COMMENTS

Background

Liver histology in sepsis has only been evaluated in animal studies and postmortem autopsies where a significant time period had elapsed between autopsy and time of death. The lack of histological data has led clinicians to evaluate sepsis-related liver damage from the serum biochemical markers while no studies have addressed the correlation between laboratory values and pathologic findings.

Research frontiers

In jaundiced septic patients, three histological patterns have been described in a limited number of studies: canalicular cholestasis, usually most severe in zone 3, ductular cholestasis with inflammation and non-bacterial cholangitis associated

with the toxic shock syndrome. Intrahepatic cholestasis in septicemia could be attributed to many factors such as circulating endotoxin causing functional disorders in bile secretion, disturbances in bile canalicular contraction and ischemia.

Innovations and breakthroughs

In order to perform a more accurate identification of sepsis related liver pathology, investigators performed liver biopsies immediately after death in fifteen septic patients. In the present study portal/lobular inflammation and/or centrilobular necrosis along with steatosis were the main findings in septic patients. Steatosis, a common finding in the post-mortem liver of septic patients was moderate to severe comprising 40%-80% of the liver parenchyma. Previous studies have shown cholestatic damage in liver parenchyma in post mortem septic specimens in contrast to the present study where cholestatic injury was present but not as frequent. The main advantage of the present study is the identification of the exact nature of sepsis related tissue abnormalities dissociated from the postmortem cellular events.

Applications

The results of the present study further add to the understanding of the pathologic changes occurring in the liver of late stage septic patients. Further studies are needed in order to examine the exact role of cellular events in septic liver.

Terminology

Portal inflammation: infiltration by inflammatory cells in portal tracts; Lobular inflammation: infiltration by inflammatory cells in hepatic lobules; Centrilobular necrosis: features of hepatocyte death in the center of lobules around terminal hepatic veins; Steatosis: accumulation of fat droplets in the cytoplasm of hepatocytes; Ductular cholestasis: accumulation of bile in bile ducts; Canicular cholestasis: accumulation of bile into bile canaliculi; cholestatic injury: damage in bile ducts due to cholestasis.

Peer review

The exact nature of liver's histological changes in septic patients described in this study thought to be the hotspot of the article.

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RAPID COMMUNICATION

Clinical significance of loss of heterozygosity for M6P/IGF2R in patients with primary hepatocellular carcinoma

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the most common type of malignancy, being especially prevalent in the Southeast Asian and sub-Saharan African populations^[1]. Etiological risk factors for HCC formation include hepatitis virus infection, alcohol consumption and dietary exposure to aflatoxin B1^[1,2]. In Korea and Taiwan, approximately 90% of all patients with HCC are Hepatitis B surface antigen (HbsAg)-positive, and prospective studies have found Hepatitis B virus carriers have a 200-fold increase in relative risk for HCC. The mannose 6-phosphate/insulin-like growth factor 2 receptor (M6P/IGF2R) is mapped at the chromosome location 6q25-27^[3], which is predicted to contain a liver tumor suppressor gene^[4]. This gene encodes a receptor which functions in intracellular lysosomal enzyme trafficking, transforming growth factor beta (TGF- β) activation, and IGF2 degradation^[5]. Granzyme B internalization by the M6P/IGF2R is also required for cytotoxic T cells to induce apoptosis in cells targeted for death, resulting in this receptor being referred to as a "death receptor"^[6]. Elevated IGF2 levels during murine development arising from M6P/IGF2R deficiency result in cardiac abnormalities, cleft palate, fetal overgrowth and prenatal lethality^[7]. Furthermore, the large-offspring syndrome found in cloned animals is frequently associated with epigenetic changes in M6P/IGF2R imprinting regulation which result in decreased gene expression^[8]. Thus, M6P/IGF2R plays a crucial role in regulating mammalian fetal growth and development. M6P/IGF2R is also mechanistically involved in the genesis of human cancer^[9-12]. M6P/IGF2R loss of heterozygosity (LOH), coupled with intragenic loss-of-function mutations in the remaining allele, is a common event in human cancers^[6,10]. Tumor cell growth is inhibited when M6P/IGF2R expression is restored to normal, whereas it is increased when gene expression is reduced^[13-16]. The results of

Abstract

AIM: To investigate the relationship between loss of heterozygosity (LOH) for mannose 6-phosphate/insulin-like growth factor 2 receptor (M6P/IGF2R) and the outcomes for primary HCC patients treated with partial hepatectomy.

METHODS: The LOH for M6P/IGF2R in primary HCC patients was assessed using six different gene-specific nucleotide polymorphisms. The patients studied were enrolled to undergo partial hepatectomy.

RESULTS: M6P/IGF2R was found to be polymorphic in 73.3% (22/30) of the patients, and of these patients, 50.0% (11/22) had tumors showing LOH in M6P/IGF2R. Loss of heterozygosity in M6P/IGF2R was associated with significant reductions in the two year overall survival rate (24.9% vs 65.5%; $P = 0.04$) and the disease-free survival rate (17.8% vs 59.3%; $P = 0.03$).

CONCLUSION: These results show M6P/IGF2R LOH predicts poor clinical outcomes in surgically resected primary HCC patients.

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Key words: Loss of heterozygosity; Mannose 6-phosphate/insulin-like growth factor 2 receptor; Hepatocellular carcinoma

these mutational and functional studies clearly show M6P/IGF2R possesses the characteristics necessary to be classified as a tumor suppressor gene^[17]. Our results show M6P/IGF2R LOH in primary HCC patients predicts poor therapeutic outcomes.

MATERIALS AND METHODS

Patients

Paraffin-embedded tissue sections from 30 patients, who were confirmed histopathologically to have HCC, were obtained from the Gyeongsang National University and the Catholic University of Korea. All patients had a history of hepatitis virus infection and/or cirrhosis, and had undergone partial hepatectomy for the treatment of their disease.

Tissue microdissection and loss of heterozygosity analysis for M6P/IGF2R

Microdissection of 10- μ m histology sections from tumor tissue and the surrounding normal liver tissue was performed as described in previous studies^[18,19]. Briefly, paraffin-embedded sections were deparaffinized in xylene (2 \times 5 min), exposed for 2 min to graded ethanol washes (namely, 100%, 95%, 70% and 50% ethanol) and rehydrated in H₂O before staining. The tissue sections were then stained for 30 s with 2% methylene blue and rinsed in H₂O before allowing them to air dry. Tumor tissue and the surrounding normal tissue (50-cells) were carefully microdissected using a serial section stained with hematoxylin-eosin for comparison. The normal tissue used for genotyping was connective tissue. The dissected tissues were then placed in 75 μ L of Tris-ethylenediamine-tetraacetic acid buffer (10 mmol/L Tris-HCl, pH 8.0 at 25°C and 0.5 mmol/L ethylenediamine tetraacetic acid, pH 8.0 at 25°C containing 5 μ L of 20 g/L proteinase K (Boehringer Mannheim, Indianapolis, IN). This mixture was incubated at 52°C for 3 h and then at 85°C for 10 min. Polymerase chain reaction (PCR) analysis was conducted using 5 μ L of this mixture, as described below. Six single nucleotide polymorphisms (Table 1), identified as c. 901C > G (exon 6), c. 1197A > G (exon 9), c. 1737G > A (exon 12), c. 2286A > G (exon 16), c. 6206A > G (exon 40) and c. X47-5t > a (exon 47), were also analyzed following 2 rounds of nested PCR. The exon-specific forward and reverse primers have been previously described^[19,20]. The exons containing these polymorphisms were amplified by PCR from genomic DNA under conditions identical to those described above. The single nucleotide polymorphisms used to determine the loss of heterozygosity in M6P/IGF2R were assessed by directly sequencing the PCR products according to the manufacturer's protocol (Thermo Sequenase, USB Corporation, Cleveland, OH). Taq DNA polymerases may introduce sequence errors during PCR amplification, and unequal amplification of the two alleles can result in the false positive detection of a loss of heterozygosity. Thus, both normal and tumor DNA templates were amplified in three independent PCR reactions, and assessed for LOH in M6P/IGF2R.

Due to the potential for contaminating the tumor tissue sample with normal stroma, allele loss in informative patients was defined as a > 50% decrease in the ratio of

the two alleles in tumor tissue *versus* that in the surrounding normal stromal tissue. This was quantified using a densitometer.

Statistical analysis

Overall survival and disease-free survival rate represented the clinical end-point. All curves were computed using the Kaplan-Meier method starting from the time of study entry. Curves for different sub-groups were compared by the Cox-Mantel test. A chi-squared test was used to compare the clinical characteristics between M6P/IGF2R-informative and M6P/IGF2R-excluded patients, and between informative patients with and without LOH in M6P/IGF2R. A $P < 0.05$ was considered to be statistically significant.

RESULTS

Analysis of LOH in M6P/IGF2R

The study population consisted of a total of 30 patients who were enrolled in a retrospective clinical trial for primary HCC from March 1999 to June 2003 (Table 2). Among the 30 patients, 22 (73.3%) were informative (that is, polymorphic), and the tumors in 50% (11/22) of these patients exhibited LOH in M6P/IGF2R (Figure 1). There was no significant difference between M6P/IGF2R-informative patients and those not used in this study in terms of the clinical characteristics of sex, age, liver cirrhosis, tumor of differentiation, size of tumor, or type of hepatitis.

Clinical outcome

The median follow-up for surviving patients enrolled in this trial was 33 mo (range: 2 to 62 mo). The median survival times were 34 mo and 23 mo for overall survival and disease free survival, respectively. There was no relationship between LOH in M6P/IGF2R and clinical factors, such as sex, age, liver cirrhosis, tumor differentiation, tumor size, or the type of hepatitis. The median overall survival times in patients with and without LOH in M6P/IGF2R were 18 mo and 44 mo, respectively, and the two year overall survival rates were 24.9% and 65.5%, respectively (log-rank, $P = 0.04$) (Figure 2A). Likewise, the median disease-free survival rates in patients with and without LOH in M6P/IGF2R were 12 mo and 36 mo, respectively, and the 3 year disease-free survival rates were 17.8% and 59.3%, respectively (log-rank, $P = 0.03$) (Figure 2B). The clinical relevance of LOH in M6P/IGF2R to both overall survival and disease-free survival rates was confirmed in the analysis ($P < 0.05$ for both comparisons). These results indicate that LOH in M6P/IGF2R results in poor patient outcome when surgical resection is employed, since all other measured clinical characteristics of the primary HCC patients were comparable to those in patients with a non-mutated M6P/IGF2R tumor suppressor gene.

DISCUSSION

M6P/IGF2R LOH occurs frequently in human breast, liver and lung cancer^[9,10,20]. Mutation in M6P/IGF2R is

Table 1 M6P/IGF2R LOH analysis of paraffin-embedded tissue

Position	Nucleotide	Genotype	Amplicon size (bp)	F1 primer (5'-3')	R1 primer (5'-3')	Nested primer (5'-3')
Exon 6	901	C/G	91	CACCAGGCGTTTGATGTTGG	CTCCAGCAAGGACCTGACTTTC	CCTCCGATGCTGTTGGCGT
Exon 9	1197	A/G	123	ACTAAGTAAGACGTGTAATCTCTAAT ACC	GTCGTGGAGAACTG AAATACAG	AATACCTATTCATATAAAACAA GCCTC
Exon 12	1737	G/A	111	TATTTGTCACAGAGTGCTGCAGG	GGCATCCAGTTTGAATGAGTTAG	GGAAGATCTAGGTGATGCTTTTC
Exon 16	2286	A/G	187	GAAGCTTTCATATTATGATGGGATG	GAGGATACTCATGCCTGTGGTG	CATCGCGCTCCCTGAGGATACT
Exon 40	6206	A/G	118	GGGTGTGATGTGACATTGTAGTGG	GCCTTCCCAGTCCACCCGC	GGAGTGCAAATTCGTCCA GAAAC
Exon 47	X47-5	t/a	161	ATGCCCTCTCTACACTGGAGTA	GTAAGCTGACCACTTG CTGTAGG	CAGTGATAAGTAAGC TGACC

Table 2 Patient characteristics *n* (%)

Characteristics	Total patients (<i>n</i> = 30)	Informative patients (<i>n</i> = 22)
Age: yr	36-78 (Median: 58)	39-78 (Median: 59)
Gender: male/female	22 (73.3)/8 (26.7)	16 (72.7)/6 (27.3)
Disease etiology		
HBV	18 (60.0)	15 (68.2)
HCV	6 (20.0)	3 (13.6)
Alcohol	6 (20.0)	4 (18.2)
Tumor Grade		
Well differentiation	9 (30.0)	6 (27.3)
Moderate differentiation	11 (36.7)	8 (36.4)
Poor differentiation	10 (33.3)	8 (36.4)
Liver Histology		
Chronic hepatitis	3 (10.0)	2 (9.1)
Cirrhosis	21 (70.0)	16 (72.7)
Nonspecific reaction	6 (20.0)	4 (18.2)
Tumor size (cm)		
< 2	4 (13.3)	2 (9.1)
2-5	14 (46.7)	11 (50.0)
> 5	12 (40.0)	9 (40.9)
No. of Tumor		
Single	18 (60.0)	12 (54.5)
Multiple	12 (40.0)	10 (45.5)
AJCC Stage		
II	18 (60.0)	12 (54.5)
III A	12 (40.0)	10 (45.5)

also commonly found in gastrointestinal and gynecological cancers, because the coding sequence of M6P/IGF2R contains a poly-G region, which is a mutational target in tumors with mismatch repair deficiencies and microsatellite instability^[12,21]. Functional studies show the introduction of an exogenous wild-type M6P/IGF2R with a single inactivated allele into human colorectal cancer cells significantly decreases cell growth rate and enhances apoptosis^[13]. Conversely, the loss of M6P/IGF2R expression promotes cancer cell growth by increasing intracellular signaling from both the receptors, the insulin-like growth factor 1 receptor and the insulin receptors^[22]. Kong *et al*^[10] demonstrated mutations in both alleles of the M6P/IGF2R are found in more than 50% of squamous cell carcinomas of the lung. In the present study, we demonstrated M6P/IGF2R LOH in primary HCC is also associated with poor patient prognosis. Loss of heterozygosity in malignancy can also occur due to chromosomal deletion or somatic recombination resulting in uniparental disomy^[23]. Because chromosomal deletion can affect more than one gene, M6P/

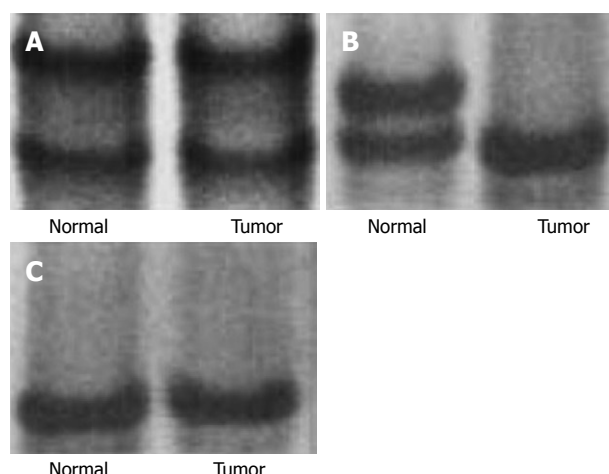


Figure 1 There are three results in the LOH analysis for M6P/IGF2R in primary hepatocellular carcinoma (HCC). A: Informative HCC without LOH in M6P/IGF2R; B: Informative HCC with LOH in M6P/IGF2R; C: Non-informative HCC.

IGF2R LOH does not rule out the possibility of a loss of adjacent genes with tumor suppressor functions in HCC. This study also showed improvements in overall survival and disease-free survival in those patients undertaking surgical resection for primary HCC with M6P/IGF2R LOH. It was found patients with mutations in M6P/IGF2R had a significantly worse prognosis than those who had a non-mutated M6P/IGF2R allele.

M6P/IGF2R is normally imprinted in mice, with only the maternal copy of the gene being expressed^[24]. By contrast, both copies of M6P/IGF2R are expressed in humans, because genomic imprinting at this locus was lost in the primate lineage approximately 70 million years ago^[25]. Importantly, the restoration of biallelic M6P/IGF2R expression in mice results in a marked reduction in offspring weight late in embryonic development that persists into adulthood^[26]. This demonstrates that M6P/IGF2R allelic loss or haploid insufficiency markedly enhances cell proliferation and/or survival during fetal development. Therefore, the mutation of even a single allele of M6P/IGF2R in human somatic cells is predicted to promote cell growth. Haploid insufficiency of tumor suppressor genes, such as Nf2, p27^{Kip1}, p53 and TGF- β , is known to promote tumor formation^[27-29]. Yamada *et al*^[9] demonstrated that, in patients chronically infected with Hepatitis B and/or Hepatitis C viruses, mutations

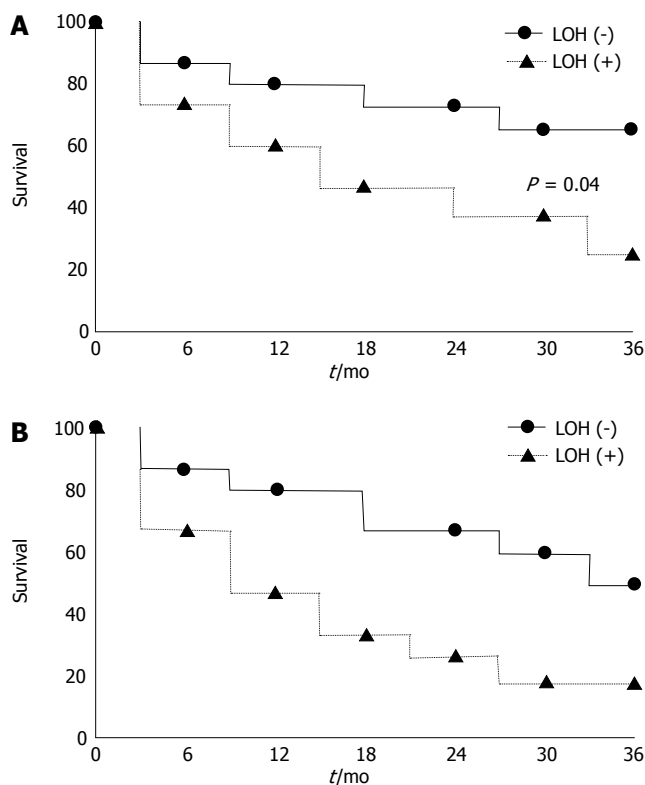


Figure 2 A: The overall survival according to loss of heterozygosity in M6P/IGF2R; B: The disease free survival according to loss of heterozygosity in M6P/IGF2R.

in M6P/IGF2R take place not only in HCC, but also in the phenotypically normal hepatocytes adjacent to these tumors. Interestingly, only one M6P/IGF2R allele is inactivated in the adjacent cirrhotic tissue, even when both alleles are mutated in the HCC. These findings are consistent with the normal appearing, preneoplastic hepatocytes forming clonal masses in the liver, because M6P/IGF2R haploid insufficiency affords them with a selective growth and/or survival advantage relative to normal hepatocytes^[30].

In conclusion, this study shows the analysis of M6P/IGF2R LOH provides clinical significance in surgically resected primary HCC patients.

ACKNOWLEDGMENTS

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COMMENTS

Background

The associated nature of hepatocellular carcinoma (HCC) and mannose 6-phosphate/insulin-like growth factor 2 receptor (M6P/GF2R) is well reported. However, there is scant research on the clinical significance of loss of heterozygosity (LOH) in M6P/GF2R in patients with primary HCC. In the present study, we aimed to investigate the relationship between LOH for M6P/IGF2R and various factors, including survival rate, in primary HCC patients treated with partial hepatectomy.

Research frontiers

Several studies have investigated various types of cancer, including HCC, in which

LOH for M6P/IGF2R might appear. We studied the relationship between LOH for M6P/IGF2R and HCC, and confirmed the survival rate is directly related to the LOH for M6P/IGF2R.

Innovations and breakthroughs

The present research studied cases with primary HCC, but research on the usefulness of LOH for M6P/IGF2R should be continued by comparing cases with metastatic HCC, cholangiocarcinoma and other tumors.

Applications

The results of this study suggest the presence of LOH for M6P/IGF2 may represent some poor prognostic factors in primary HCC patients treated with hepatectomy.

Peer review

The paper represents a real advance in the loss of heterozygosity for M6P/IGF2R in patients with primary HCC. The conclusions are valuable. The methodology is correct and the results are well presented.

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Intestinal permeability and its association with the patient and disease characteristics in Crohn's disease

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Abstract

AIM: To assess the intestinal permeability (IP) in patients with Crohn's disease (CD) and study the association of IP with the patient and disease characteristics.

METHODS: One hundred and twenty five consecutive patients of CD (Males: 66) were diagnosed on the basis of a combination of standard clinical, endoscopic, imaging and histological features. CD activity index (CDAI) was used to calculate the activity of the disease while the behavior of the disease was assessed by the modified Montreal classification. IP was measured by the ratio of the percentage excretion of ingested doses of lactulose and mannitol in urine (LMR). The upper limit of normality of LMR (0.037) was derived from 22 healthy controls.

RESULTS: Thirty six percent of patients with CD had increased IP. There was no significant difference in mannitol excretion (patients *vs* controls = 12.5% *vs* 14.2%, $P = 0.4652$), but lactulose excretion was significantly higher in patients compared to healthy controls (patients *vs* controls = 0.326% *vs* 0.293%, $P = 0.0391$). The mean LMR was also significantly higher in the patients as compared to healthy controls [0.027 (0.0029-0.278) *vs* 0.0164 (0.0018-0.0548), $P = 0.0044$]. Male patients had a higher LMR compared to females [0.036 (95% CI 0.029, 0.046) *vs* 0.022 (95% CI 0.0178, 0.028) ($P = 0.0024$), though there was no difference in the number of patients with abnormal IP in both

the sexes. Patients with an ileo-colonic disease had a higher LMR than those with only colonic disease [0.045 (95% CI 0.033, 0.06) *vs* 0.021 (95% CI 0.017, 0.025) ($P < 0.001$)]. Of patients with ileo-colonic disease, 57.8% had an abnormal IP, compared to 26.7% with colonic and 15.6% with small intestinal disease. Patients with a stricturing disease had significantly higher LMR compared to non-fistulising non-stricturing disease [0.043 (95% CI 0.032, 0.058) *vs* 0.024 (95% CI 0.019, 0.029) ($P = 0.0062$)]. There was no correlation of IP with age, disease activity, duration of illness, D-xylose absorption, upper GI involvement, perianal disease, and extra-intestinal manifestations. On multiple regression analysis, male gender and ileo-colonic disease were independent factors associated with increased IP. Gender, location, behavior of the disease and upper GI involvement could explain up to 23% of variability in IP ($R^2 = 0.23$).

CONCLUSION: IP was increased in 36% of patients with CD. Male gender and an ileo-colonic disease were the independent factors associated with increased IP.

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Key words: Lactulose mannitol ratio; Crohn's disease; Inflammatory bowel disease; Intestinal barrier; Crohn's disease activity index; Intestinal permeability

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INTRODUCTION

Intestinal permeability (IP) is the property of the intestinal epithelium which refers to the facility with which it allows molecules to pass through by non-mediated diffusion^[1]. IP has been implicated in the pathogenesis and frequent relapses of Crohn's disease (CD)^[2-6]. Seven to 18% higher relapse rate has been reported in patients with increased IP compared to those with normal IP^[7-11]. Moreover,

10%-20% of healthy first-degree relatives have been also shown to have increase IP^[4,12,13]. IP is measured as a ratio of two non-metabolizable probe molecules that pass across the mucosal barrier and are excreted in the urine. Quantitation of these probes in a timed urine collection provides a measure of the fraction of the ingested dose that penetrated the mucosal barrier^[14]. The individual variations due to non-mucosal factors (gastric emptying, intestinal transit, dilution by secretions, renal clearance and incomplete urine recovery) are circumvented when the urinary recovery is expressed as a ratio, since both the sugars are equally affected by these factors except the route of permeation^[15]. A few studies have reported the association of IP with activity and location of Crohn's disease; however, there is a lack of literature on the association of IP with the disease characteristics (duration, extent, behavior, extra-intestinal manifestations) and patient characteristics (age and gender). There are no reports on IP in Asian patients with CD. Hence, this study was planned to assess IP in patients with CD and also to explore the relationship of IP with the patient and disease characteristics.

MATERIALS AND METHODS

Patients

One hundred and twenty five consecutive patients with CD from the outpatient and inpatient of Gastroenterology Department, All India Institute of Medical Sciences, New Delhi, were studied between May 2005 and September 2006. The diagnosis of CD was made on the basis of clinical manifestations (chronic diarrhea, hematochezia, abdominal pain and intestinal obstructive symptoms), endoscopic features (skip lesions, asymmetrical involvement, deep ulcers, aphthous ulcers, ileocecal valve and terminal ileal involvement) and histological evidence (acute on chronic colitis, inflammation extending beyond muscularis mucosa, lymphoid follicles and non caseating granuloma). The involvement of small intestine was assessed by barium meal follow through, small bowel enema, magnetic resonance enteroclysis and/or retrograde ileoscopy. Disease activity was assessed using the Crohn's disease activity index (CDAI)^[16]. The location and behavior of the disease were classified using the modified Montreal classification^[17]. Patients were treated with maintenance doses of mesalamines and azathioprine, along with hematinics, multivitamins and calcium supplements. Those in the active phase of the disease were treated with steroids. None of the patients in active phase of the disease had any evidence of bacterial or parasitic infection at the time of inclusion into the study.

Healthy controls

Twenty-two healthy controls comprising of hospital staff and family members of patients with a diagnosis other than inflammatory bowel disease were included to decide the upper limit of normality (cut-off) for lactulose mannitol ratio (LMR). None of the controls had signs and symptoms of gastrointestinal disorders; renal diseases, diabetes and none of them had taken NSAIDs for at least

four weeks prior to the test. None had a history of alcohol intake and smoking.

Ethical Consideration: The Ethics Committee of our institution approved the study. An informed consent was taken from all the participants.

Assessment of intestinal permeability

IP was measured using the lactulose and mannitol (L:M) excretion test in all the patients and healthy controls. The results were expressed as the ratio of percentage excretion of the ingested dose of lactulose and mannitol in urine [lactulose mannitol ratio (LMR) = % lactulose/% mannitol].

Test procedure

After an overnight fast, the patients evacuated the urinary bladder, collected a pre-test sample and then drank the test solution containing 5 g of lactulose, 2 g of mannitol and 5 g of D-xylose in 100 mL water. No food or drink other than water was allowed until the completion of the test. Water was permitted after one hour of the ingestion of the test solution. All the urine passed in the subsequent five hours was collected into a plastic can containing 20% chlorhexidine as a preservative. Aliquots of the collected urine were stored at -20°C until analysis. Patients were instructed to abstain from NSAIDs for four weeks prior to the test. All the patients and controls tolerated the test solution well.

Estimation of mannitol in urine

This test was based on the principle of oxidation of mannitol to formaldehyde by periodic acid. The formaldehyde so produced was measured by the method of MacFadyen as described by Corcoran and Page^[18].

Periodic acid (0.03 mol/L in 0.25 mol/L sulfuric acid; 500 µL) was added to 2 mL of urine specimen in a test tube and kept at room temperature for 10 min. Stannous chloride (0.125 mol/L; 500 µL) was added to the sample and mixed well. Oxidation of stannous chloride to stannic acid produced a milky precipitate, which was dissolved by adding 5 mL of chromotropic acid reagent. The tubes were then placed in a boiling water bath for 30 min. After cooling the tubes the volume of the solution was made up to 25 mL by adding distilled water and the temperature was stabilized at 25°C in a water bath. A reagent blank was prepared containing 2 mL of distilled water instead of sample and was treated in the same manner along with the sample tubes. The optical density was measured at 570 nm using a spectrophotometer^[18].

Estimation of lactulose in urine

The estimation of lactulose was based on the enzymatic assay of fructose which was produced after the hydrolysis of lactulose to fructose and galactose. In a series of enzymatic reactions, fructose was converted to NADPH. The amount of NADPH produced was directly proportional to lactulose concentration, which was measured by the change in absorbance at 340 nm on a spectrophotometer^[19]. D-xylose was estimated in urine using the standard method of Roe and Rice^[20].

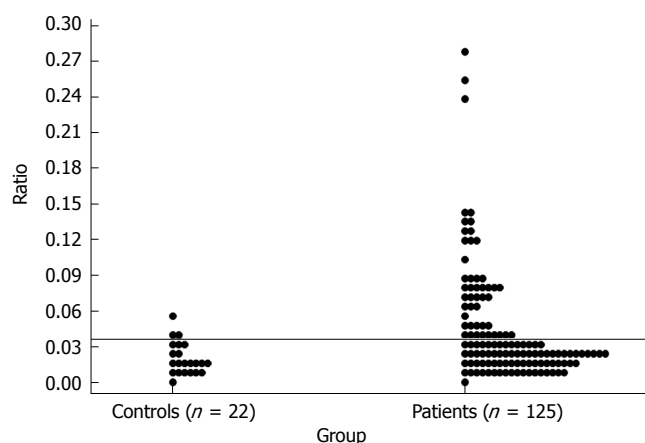


Figure 1 Upper limit of normality for lactulose mannitol ratio (LMR).

Estimation of the cut-off for the LMR

The results of the LMR in the healthy controls were used to fix the upper limit of IP at one tailed 90% tolerance interval. Since the distribution of LMR in controls was non-normal, a square root-transformation was carried out to determine the mean and standard deviation (SD) and subsequently the cut-off value was established as mean + 1.28 SD of the square root of LMR, assuming that in normal population 10% subjects will have abnormal IP. Thus obtained cut off value would correspond to the 90th percentile. A LMR of > 0.0373 was considered abnormal (Figure 1).

Statistical analysis

STATA 9.0 (College station, TX, USA) statistical software was used for data analysis. The data were expressed as number (percentage), median (range) and mean (95% CI). The difference in proportions of abnormal IP between different categories of disease area, behavior, activity and gender were compared using χ^2 test. Non-normal continuous variables were compared between the patients and controls using non-parametric test (Wilcoxon rank-sum test). Bivariate analysis was done using *t*-test, ANOVA or Spearman's correlation as appropriate followed by multiple regression analysis to find the factors related with increased IP. The *P*-value < 0.05 was considered statistically significant.

RESULTS

The median (range) age of 125 patients was 36 (14-66) years. The median (range) duration of the disease was 50 (3-432) mo. The duration of the disease was less than 36 mo in 52 (41.6%) and more than that in 73 (58.4%) patients. The distribution of the patients according to activity, duration, location, and behavior of the disease is shown in Table 1.

Twenty three (18.4%) patients had involvement of the UGI tract, of which 6 (26.08%) had ileal, 6 (26.08%) had colonic and 11 (47.8%) had ileo-colonic involvement.

Lactulose and mannitol excretion and LMR in patients with CD

The percentage excretion of lactulose and mannitol, values

Table 1 Characteristics of the patients with CD (*n* = 125)

Variables	<i>n</i> (%)
Sex	
Males	66 (52.8)
Females	59 (47.2)
Age (yr)	
Median (range)	36 (14-66)
Patients with age < 40	76 (60.8)
Patients with age ≥ 40	49 (39.2)
Duration of the disease (mo)	
Median (range)	50 (3-432)
Duration < 36 mo	52 (41.6)
Duration ≥ 36 mo	73 (58.4)
Activity of the disease (CAAI)	
Median (range)	109 (10-343)
CAAI < 150	80 (64)
CAAI 151-220	29 (23.2)
CAAI 221-400	16 (12.8)
CAAI > 400	0 (0)
Area of involvement of the GI tract	
Small intestine only (L1)	16 (12.8)
Large intestine only (L2)	62 (49.6)
Ileo-colonic (L3)	47 (37.6)
UGI involvement (L4)	23 (18.4)
Behaviour of the disease	
Non-fistulizing non-stricturing (B1)	74 (59.2)
Stricturing disease (B2)	37 (29.6)
Fistulizing disease (B3)	14 (11.2)
Perianal disease	25 (20)
Extra-intestinal manifestations	49 (39.2)

CD: Crohn's disease; CDAI: Crohn's disease activity index.

Table 2 Comparison of intestinal permeability and D-xylose absorption between patients and healthy controls

Parameter	Patients (<i>n</i> = 125)	Controls (<i>n</i> = 22)	<i>P</i> value
Lactulose excretion (%)	0.326 (0.0204-2.76)	0.293 (0.0089-0.665)	0.0391 ^a
Mannitol excretion (%)	12.5 (1.43-43.75)	14.2 (4.95-30.8)	0.4652
Lactulose mannitol ratio (LMR)	0.027 (0.0029-0.278)	0.0164 (0.0018-0.0548)	0.0044 ^a
D-xylose (g/5 g per 5 h)	1.45 (0.32-4.5)	1.89 (0.80-4.73)	0.0277 ^a

All values expressed as median (range); ^a*P* < 0.05.

of LMR and D-xylose excretion are shown in Table 2. Using the upper limit of normality for LMR at 0.0373, 45 of 125 (36%) patients had an abnormal IP, while 80 (64%) had a normal IP.

Lactulose, mannitol and D-xylose excretion in healthy controls and patients with CD

As shown in Table 2 there was no significant difference in percentage excretion of mannitol in patients and healthy controls (*P* = 0.4652). The percentage excretion of lactulose was significantly higher in patients compared to healthy controls (*P* = 0.0391). Similarly, the LMR was higher in the patients compared to healthy controls (*P* = 0.0044). D-xylose excretion was also significantly low in patients compared to healthy controls (*P* = 0.0277).

Table 3 Relationship between intestinal permeability (log LMR) and characteristics of the patients and disease ($n = 125$)

Variables	<i>n</i>	Mean LMR (95% CI)	<i>P</i> value
Gender			
Males	66	0.036 (0.029, 0.046)	0.0024 ^a
Females	59	0.022 (0.0178, 0.028)	
Area of involvement of the GI tract ^b			
Small intestine only (L1)	16	0.028 (0.0175, 0.0447)	0.0001 ^a
Large Intestine only (L2)	62	0.021 (0.0174, 0.0255)	
Ileo-colonic (L3)	47	0.0451 (0.0336, 0.0605)	
UGI Involvement			
Absent	102	0.0268 (0.0224, 0.0321)	0.0406 ^a
Present	23	0.0416 (0.027, 0.062)	
Perianal disease			
Absent	100	0.028 (0.023, 0.033)	0.3573
Present	25	0.0339 (0.0234, 0.049)	
Behavior of the disease ^d			
Non-fistulizing	74	0.024 (0.0194, 0.0298)	0.0062 ^a
Non-stricturing (B1)			
Stricturing (B2)	37	0.043 (0.032, 0.058)	
Fistulizing (B3)	14	0.028 (0.018, 0.042)	
Extra intestinal manifestations			
Absent	76	0.028 (0.0226, 0.034)	0.5674
Present	49	0.030 (0.0236, 0.040)	

Values are expressed as geometric mean (95% CI), ^a $P < 0.05$; Large intestine only *vs* ileo-colonic, ^b $P = 0.001 < 0.01$; Non-fistulizing non-stricturing *vs* stricturing, ^d $P = 0.004 < 0.01$.

Table 4 Relationship of LMR with the patient and disease characteristics using Spearman's correlation coefficient

Variables	<i>r</i>
Age (yr)	-0.1414
Activity of the disease (CDAI)	0.1414
Duration of the disease (mo)	-0.0271
D-xylose (g/5 g per 5 h)	-0.1210

Relationship of LMR with patient and disease characteristics

As shown in Table 3, male patients had a significantly higher LMR in comparison to that in females ($P = 0.0024$). There was no difference in the number of patients with abnormal IP in both the sexes ($P = 0.05$). There was no significant correlation between IP and age of the patients (Table 4).

LMR and location of the disease: On comparison of LMR in patients with different location of the disease (L1, L2, L3), a significant ($P = 0.0001$) difference was found. Patients with ileo-colonic involvement had a higher LMR in comparison to those with only colonic involvement ($P = 0.0001$). Higher percentage (57.8%) of patients with ileo-colonic disease had abnormal IP compared to the colonic (26.7%) and small intestine (15.6%) disease, which was statistically significant ($P < 0.001$).

LMR and behavior of the disease: There was a significant difference ($P = 0.0062$) in the LMR of patients with different behavior of the disease (B1, B2, B3). Patients with stricturing disease (B2) had significantly higher LMR in comparison to those with a non-fistulizing and non-

Table 5 Assessment of the effect of disease and patients characteristics on log LMR using multiple regression analysis

Variable	Regression co-efficient β (95% CI)	
	Unadjusted	Adjusted
Sex		
Females ¹		
Males	0.39 (0.089, 0.69)	0.429 (0.130, 0.727) ^a
Area		
Small intestine only ¹		
Large intestine only	-0.284 (-0.764, 0.196)	0.002 (-0.539, 0.543)
Ileo-colonic	0.475 (-0.020, 0.971)	0.579 (0.065, 1.09) ^a
Behaviour		
Non-fistulizing non-stricturing ¹		
Stricturing disease	0.587 (0.230, 0.945)	-0.237 (-0.763, 0.288)
Fistulizing disease	0.152 (-0.366, 0.670)	0.085 (-0.457, 0.628)
Upper GI involvement		
Absent ¹		
Present	0.437 (0.019, 0.856)	0.233 (-0.163, 0.630)

$R^2 = 23\%$, ^a $P < 0.05$, significant sqrt-square-root transformation. ¹Reference category; β : Regression coefficient; CI: Confidence interval.

stricturing disease ($P = 0.004$). Higher percentage (46.7%) of patients with a stricturing disease had abnormal IP compared to the non-stricturing non-fistulizing (42.2%) and fistulizing (11.1%) disease ($P = 0.006$).

LMR and UGI involvement, perianal disease and extra-intestinal manifestations: There was no significant difference in the LMR of the patients with and without extra-intestinal manifestations and perianal disease ($P > 0.05$). However, patients with an UGI involvement had a significantly higher LMR as compared to those without an UGI involvement ($P = 0.046$).

LMR and activity and duration of the disease: Twenty eight of 83 (33.7%) patients in the remission phase and 17 of 42 (40.47%) in the active phase of the disease had increased IP, however, it was not statistically significant. Spearman's correlation did not show any association of LMR with activity and duration of the disease (Table 4).

Factors associated with increased IP

Sex, area, behavior and UGI involvement were the significant factors on a bivariate analysis. A multiple regression analysis revealed that all these factors could explain up-to 23% of variability in IP ($R^2 = 0.23$) (Table 5). Out of all these factors gender of the patients and ileo-colonic disease were independent factors associated with increased IP.

On an average, log LMR value among males was 0.429 (95% CI 0.130, 0.727) more as compared to log LMR value in females. Patients with ileo-colonic disease on an average had a 0.58 (95% CI 0.065, 1.09) log units increase in the LMR compared to those with a small intestine disease (Table 5). Thus, male sex and ileo-colonic disease were found to be a risk factor for increased IP in patients with CD.

DISCUSSION

In our study, 36% of patients with CD had increased

intestinal permeability. Males, patients with ileo-colonic disease, stricturing disease and those with UGI tract involvement had a significantly higher IP than females, patients with colonic disease, those with non-stricturing non-fistulizing disease and those without the UGI tract involvement, respectively. All these factors together could explain up to 23% of variability in IP. Male sex and ileo-colonic location of the disease were the independent factors associated with increased IP. However, there was no correlation of IP with age, duration, activity of the disease and D-xylose absorption.

Thirty three to 68% patients with CD have been reported to have increased IP using a variety of marker probes such as polyethylene glycol (PEG) 400, PEG 1000, $^{51}\text{CrEDTA}$, $^{99\text{m}}\text{TcDTPA}$, $^{51}\text{CrEDTA}/^{14}\text{C}$ -mannitol, lactulose, mannitol, rhamnose and cellobiose^[21-24]. In this study, we used lactulose and mannitol (L/M) excretion to quantify IP. L/M test is a simple, non-invasive, reliable and a frequently used test for estimation of IP in clinical practice^[25,26]. Our study showed that IP, as assessed by LMR, was significantly higher in patients compared to the healthy controls. There was no difference in the mannitol excretion amongst patients and controls, but the excretion of lactulose, was significantly higher in patients as compared to controls. Therefore, an abnormal LMR was due to higher excretion of lactulose in the urine than a reduced excretion of mannitol. Pearson *et al*^[15], Murphy *et al*^[3], Wyatt *et al*^[7], and Katz *et al*^[27] have also reported similar findings. The probes used to measure IP are water soluble, which cannot penetrate the lipid cell membrane of enterocytes and thus use the paracellular route through the tight junctions (TJs) for permeation. There is a difference between the TJs of the villous tips and villous crypts. The smaller probes can easily pass through the small, more numerous and more accessible TJs of the villous tips, whereas the larger probes make use of the larger, less accessible, and less numerous pores at the crypt of the villous^[28,29]. While mannitol uses predominantly smaller and more numerous TJs at the villous tips, lactulose being a bigger molecule passes through larger pores in the crypts of the villous. As patients with CD do not generally have diffuse villous atrophy, the mannitol excretion remains unaffected in them and this could explain a normal mannitol excretion values observed in this study. Contrary to this, villous atrophy is the hallmark of celiac disease, which results in loss of smaller TJs at the villous tips, thus affecting excretion of mannitol^[15,30]. Therefore, an increased IP observed in patients with CD, is predominantly because of increased permeation of lactulose, possibly due to defective TJs at the crypt. Nonetheless, one study has shown a decreased mannitol excretion in patients with CD as compared to the healthy controls^[31], where reduced mannitol absorption reflects a reduction in the absorptive area of mucosa rather than change in mucosal leakiness.

We did not find any correlation between IP and age of the patients. Similar findings have been shown by Soderholm *et al*^[23]. While Munkholm^[32] and Jogerson *et al*^[21] found a negative correlation of age with the absorption of mannitol and excretion of $^{51}\text{CrEDTA}$, respectively.

Male sex was found to be a risk factor for increased IP on a multiple regression analysis in this study. Jorgensen *et al* reported an association between the male gender and increased permeation of ^{14}C -mannitol^[21]. On the contrary, Soderholm *et al*^[23] did not find any correlation between IP and gender of the patients with CD. The reason of higher IP in male patients is not clear. We presume that it may be attributed to some other environmental factors, like consumption of tobacco and alcohol which was only prevalent among males in our study.

We also observed a significantly higher IP in patients with ileo-colonic disease as compared with those with only a colonic disease. Ukabam *et al*^[31] and Ainsworth *et al*^[33] have also reported a significantly increased IP in patients with ileal CD than those with colonic CD using L/M ratio and $^{51}\text{CrEDTA}$, respectively. Peters *et al*^[22] reported a significantly higher median permeability of $^{51}\text{Cr EDTA}$ in patients with ileal CD compared to normal controls; no significant difference was seen in patients with colonic CD and normal controls. The possible reason that could be given in support of our findings is the choice of marker probes. Different marker probes with regional selectivity are used to study IP in different locations of GI tract. Sucrose is used for gastric and proximal duodenal permeability; lactulose and mannitol for small intestine permeability; sucralose, poly sucralose and EDTA for both large and small intestine permeability^[28]. Nevertheless, most of the probes are much better in detecting the permeability abnormalities of the small intestine than that of the colon^[34]. Moreover, mannitol permeability does not differentiate between CD of the small intestine and large intestine. Abnormal IP in patients with ileo-colonic involvement in this study may be a reflection of selection of a marker probe, which assessed the small intestinal permeability only. In a few studies on the other hand, no relation was observed between IP and location of the disease^[23,27,35]. No association was found between IP and perianal disease.

It was also seen that patients with a stricturing disease had a significantly higher LMR than a non-fistulizing non-stricturing disease. Similar observations have not been reported in the English literature to the best of our knowledge. We also hypothesize that an abnormal IP might lead to frequent and prolonged relapses, which in turn may have lead to the development of intestinal strictures.

Increased IP, molecular mimicry, translocation of antigens and circulating cytokines are the proposed pathogenic mechanisms of extra-intestinal manifestations in patients with CD^[36]. We, however, did not observe an association between increased IP and extra-intestinal manifestations in this study.

A number of studies have reported a relationship between abnormal IP and the activity of the disease^[3,5,37,38]. In some reports it has been observed that the magnitude of alterations in L/M ratio increases with the increase in disease activity index. In fact, assessment of IP has been recommended as a more objective way of assessing the activity and severity of the disease^[39]. But, we did not find any correlation between L/M ratio and activity of the disease. Though there were a greater number of patients

with abnormal IP in the active phase of the disease than in the remission phase, this did not, however, reach a value of statistical significance. Ukabam *et al*^[31] and Soderholm *et al*^[23] also did not observe a relationship between IP and activity of the disease. Similarly, Turck *et al*^[35] reported that the highest excretion values of ⁵¹Cr EDTA were not always observed in children with the most active CD.

In conclusion, we observed increased intestinal permeability in 36% of patients with CD and this was mainly because of the increase in the excretion of lactulose. Male gender and ileo-colonic involvement were independent risk factors for increased intestinal permeability in our patients.

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COMMENTS

Background

Intestinal permeability (IP) is an important aspect of the gut barrier function of the intestinal mucosa. Its role has been emphasized recently in a number of conditions including Crohn's disease (CD). In case of CD it has been implicated in its etiopathogenesis, assessment of the disease activity, and a predictor of relapse. The incidence of CD has been recently reported to be on a rise in the Asian countries including India. Hence, we decided to study IP and its correlation with the patient and disease characteristics in Crohn's disease in India

Research frontiers

There are a number of studies from the western world on the study of IP. It has been reported that IP may be abnormal even in those with a macroscopically normal gut. It is believed that IP is maintained by the tight junctions (TJs). It will be interesting to further study that apart from atrophy of the mucosal surface what else makes the TJs ineffective. Probability of some defect or abnormality in TJs proteins has been suggested for the abnormal IP. It will be further interesting to know the characterization of these proteins and then improve upon them by some intervention

Innovation and breakthroughs

There are several studies reporting abnormal IP not only in CD, but also in blood relations of the patients. There is also a case report where a patient with abnormal IP ultimately developed CD. There have been no reports on IP in Asian patients with CD. Hence this study is an initial attempt in this direction to study IP and identify those patients & disease characteristics which might influence IP in patients with CD.

Application

Our study reveals that up to 36% of patients have abnormal IP. This shows that IP in Indian patients is the same as that of the western population. We thought that India, being a tropical country, might have greater prevalence of abnormal IP than the western world. The gender difference shown in IP in our patients signifies that there may be a variation in the presentation and clinical course of the disease in both the sexes, as has been reported in pediatric patients with CD.

Terminology

Intestinal permeability (IP): IP can be defined as the property of the intestinal epithelium or of a membrane which refers to the facility with which it allows molecules to pass through by non-mediated diffusion. Tight Junctions (TJs): The tight junctions encircle epithelial cells at the apical pole, being a narrow belt that

both connects adjacent cells and maintains cell polarity. TJs represent the gating mechanism in the paracellular pathway.

Peer review

This research adds to the growing body of literature regarding intestinal permeability in a variety of pathogenic states including inflammatory bowel disease. It suggests that the underlying mucosal defect is at the crypt and not at the villous level and thus it yields a new level of understanding to the underlying mechanism.

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RAPID COMMUNICATION

Scintigraphic evaluation of gallbladder motor functions in *H pylori* positive and negative patients in the stomach with dyspepsia

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CONCLUSION: Our study showed that ^{14}C -UBT is highly reliable method to detect the presence of *H pylori*. The presence of *H pylori* infection does not directly affect the GB function.

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Key words: *H pylori*; Dyspepsia; Cholescintigraphy; Gallbladder; ^{14}C -urea breath test

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Abstract

AIM: To evaluate the relationship between gallbladder (GB) motor function and *H pylori* infection in the stomach.

METHODS: All cases (86) underwent the ^{14}C urea breath test (UBT). ^{14}C -UBT was found as positive in 58 and negative in 28 dyspeptic patients. ^{14}C -UBT was accepted as a gold standard test. Clo test and histopathologic examination were compared with the results of ^{14}C -UBT in cases who tolerated upper gastrointestinal endoscopy procedure. Cholescintigraphy with $^{99\text{m}}\text{Tc}$ -mebrofenin was used to determine the parameters of GB motor function (GB filling and emptying time, half of the emptying time, ejection fraction at 30th and 60th min) in all patients.

RESULTS: We found the sensitivity and specificity as 88% and 86% for Clo test and as 89% and 80% for histologic evaluation, respectively. The parameters of GB function were not significantly different in *H pylori* positive and negative patients. The GB emptying was normal in both groups. Minimum GB filling time was 30 min in 34 of 86 cases (39.5%), filling was not observed in 2 cases. The GB ultrasonography (USG) results were normal for all cases and bile composition abnormality was not determined.

INTRODUCTION

The *H pylori* infection is one of the most common chronic infections in humans^[1,2]. The *H pylori* colonizing on the surface of the upper gastrointestinal mucosa is an interesting cause of active chronic gastritis and duodenitis or even gastric cancer worldwide^[2-7]. The presence of *H pylori* infection could predispose to various disorders^[8-14]. Dental disease might be associated with a higher recurrence of *H pylori* infection^[9]. Some investigators have recently demonstrated the evidences that *H pylori* infection induced atherosclerosis and that *H pylori*-anti-heat-shock protein antibodies have been related to the prevalence of diseases such as coronary artery disease or cerebral infarction, resulted from atherosclerosis^[10,11]. The *H pylori* may play a role in the pathogenesis of slow coronary flow *via* the elevation of homocysteine, and/or a possible disturbance in its metabolism^[12].

The *H pylori* is present in about 67%-100% of duodenal ulcer patients and 13%-61% of normal population^[13]. The *H pylori* infection can be diagnosed by invasive techniques requiring endoscopy and biopsy (eg, histological examination and culture) and by noninvasive techniques such as serology, urea breath test or detection of *H pylori*

antigen in stool specimen^[1,16]. Urea breath tests (UBT) are based on the principle that urease activity is present in the stomachs of individuals infected with *H pylori*. The UBT is considered as the gold standard in the diagnosis of *H pylori* among the noninvasive tests^[16-19].

Gallbladder (GB) diseases are related more to an emptying abnormality than to resting volume changes^[20]. Cholescintigraphy using ^{99m}Tc-hepatobiliary iminodiacetic acid (HIDA) is a scintigraphic technique for measuring GB emptying. Cholescintigraphy is used to show both morphological and physiological changes in GB. Since physiological changes usually precede morphological alterations by several weeks or months, there is a great potential for early diagnosis by scintigraphy, before irreversible functional changes take place. The main advantage of cholescintigraphy is that the technique is noninvasive, quantitative, reproducible and has a low interobserver error^[20-24]. Dynamic biliary scintigraphy can measure biliary motor functions noninvasively and quantitatively in *H pylori* positive and negative patients with dyspepsia^[22]. Few reports have been published on the relationship between gallbladder emptying in patients with *H pylori* positive and negative idiopathic dyspepsia^[22,26]. Studies performed on this topic have not shown that dyspepsia could be related to gallbladder function.

The association between GB emptying and gastric emptying in dyspeptic patients has been investigated with several techniques such as real time ultrasonography, scintigraphy for gastric emptying, hepatobiliary scintigraphy and the emptying time was found similar for both of them^[25,26]. There is no literature available to confirm the effects of *H pylori* on the GB and gastrointestinal motor functions and regarding the relationship between these functional disorders and dyspeptic symptoms.

The objective in our study was scintigraphic evaluation of GB motor functions in *H pylori* positive and negative patients affected by dyspepsia.

MATERIALS AND METHODS

Patients and data collection

The study included 86 patients (44 male, 42 female) with symptoms of dyspepsia. These patients had no other complaints or systemic diseases and were not on any medications. The permission of the study was given by local ethic committee.

The *H pylori* was determined in each patient with ¹⁴C-UBT (Isotopes Co. Ltd. institute, Noster Sys. AB, HELIPROBE) as a gold standard as it is noninvasive, easy to perform and cheap diagnostic method for *H pylori*. Before the ¹⁴C-UBT, the subject fasted for at least 6 hours. *H pylori* positive 58 patients (23 female, 35 male; mean age 41 years) as study group and *H pylori* negative 28 subject (19 female, 9 male; mean age 45 years) as control group were studied. Written informed consent was obtained for each patient. The subjects were given ¹⁴C-urea capsule orally with 20 mL water. After 15-30 min, the subject exhaled into the BreathCard. The average breathing time was approximately 1-2 min. The Heliprobe BreathCard was put into slot of the Heliprobe Analyzer and after pressing start button, the

analyzer was measured within 4 min. The analysis is based on the number of emitted β -particles and is presented as decay per min (DPM) together with the test result < 50 DPM (0, negative), 50-199 DPM (1, equivocal), and \geq 200 DPM (2, positive). Within one week, the urease test (Clo test) and histopathologic examination were compared with the results of ¹⁴C-UBT in cases who tolerated upper gastrointestinal endoscopy procedure. Upper gastrointestinal endoscopy (Olympus GIF 1T 30) with biopsies from antrum and corpus was performed in total of 74 cases (52 patients and 22 control subjects) after history and physical examination were obtained. Twelve cases (6 patients and 6 control subjects) could not tolerate endoscopic examination.

After ¹⁴C-UBT, every patient was defined by using cholescintigraphy with ^{99m}Tc-mebrofenin to determine the parameters of GB motor function.

Dynamic Cholescintigraphy

After 6-8 h of fasting, all patients were injected intravenously 5 mCi of ^{99m}Tc Mebrofenin (NYCOMED AMERSHAM SORIN S.r.l., BRIDATEC) while lying supine underneath a gamma camera fitted with a 140-keV low energy, all purpose, parallel-hole (LEAP) collimator. The gamma camera (GE- Milenium Acq, entegra) was connected to a computer, which enabled simultaneous data acquisition in a 128 \times 128 matrix. Dynamic acquisition was started at time 0 min with simultaneous administration of a bolus injection of ^{99m}Tc Mebrofenin and was obtained (15 s/frame) for 5 min. After this acquisition, gallbladder filling was observed for approximately 30-60 min and at max. filling time, orally a standard fatty meal (100 g milky chocolate) instead of CCK (sincalide, kinevac) was ingested in the sitting position to stimulate gallbladder emptying. This has provided a physiological stimulation of GB contraction and prevented the false positive results. During the following 15 min, dynamic acquisition was started while lying supine with 30 s/frame and acquisition continued for 60-90 min thorough GB emptying (Figures 1 and 2).

All of the summed dynamic images (before and after oral stimulation of GB emptying) were evaluated with the raw data and cine projections from the computer.

We calculated the following parameters to describe GB emptying: (1) The filling time of gallbladder (GBFT): Time (min) for maximum counts per min during the interval between the filling period of gallbladder and meal ingestion; (2) Gallbladder Ejection Fraction (GBEF) at 30 min and 60 min; (3) Gallbladder half emptying time (GB 1/2): This parameter was calculated automatically from the time-activity curve on the computer (GE Entegra).

To determine interobserver variation, GBEF (for 30 min and 60 min) was calculated independently by two separate observers (experienced and inexperienced nuclear medicine physicians) at separate times.

Statistical analysis

Data were analyzed with the SPSS 10.0 program. Statistical analysis was performed by using student's *t* test and *P* < 0.05 was considered as statistically significant. The data was presented as mean \pm SE or as mean \pm SD.

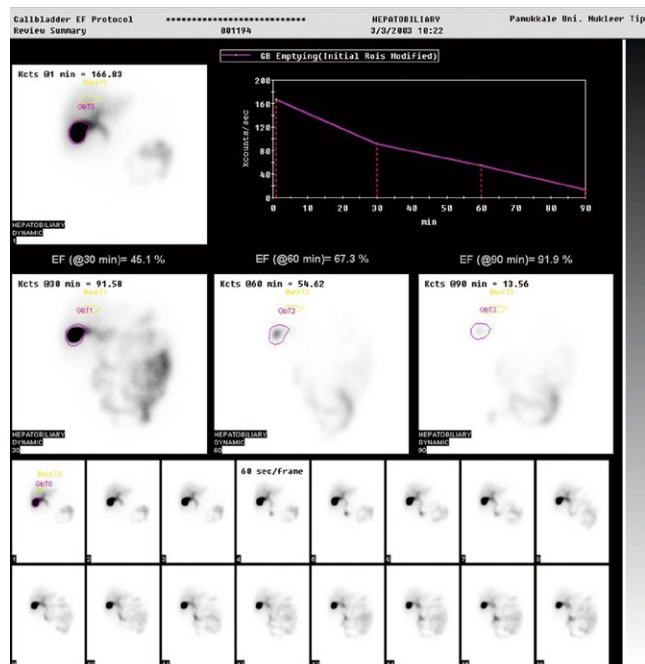


Figure 1 The image of a normal ^{99m}Tc-mebrofenin cholescintigraphy. Emptying of the whole gallbladder content takes almost 90 min.

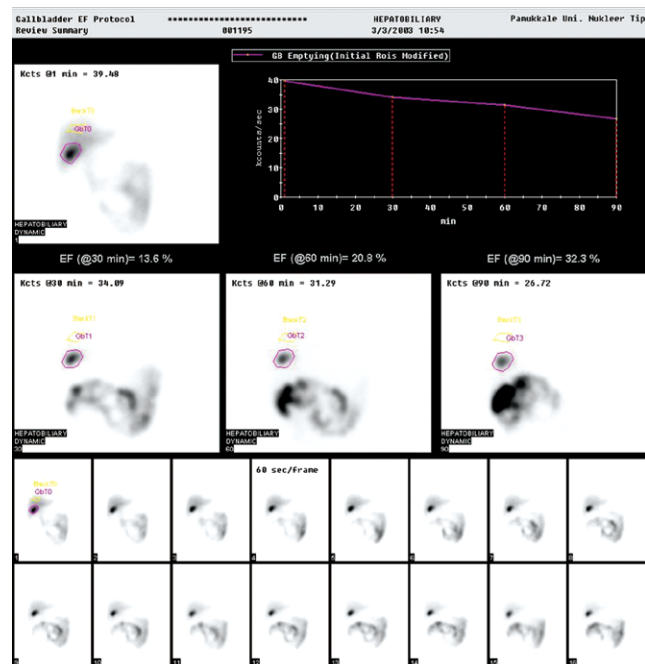


Figure 2 Emptying of the gallbladder content was slower and did not complete during the study in case with impaired GB functions.

Table 1 Gallbladder motor function parameters in *H pylori* positive and negative patients (mean ± SE)

¹⁴ C-UBT	GBFT (min)	GB t1/2 (min)	GBEF ₃₀ (A) %	GBEF ₆₀ (A) %	GBEF ₃₀ (B) %	GBEF ₆₀ (B) %
Positive (n = 58)	53.71 ± 3.49	44.15 ± 4.38 (n = 27)	36.58 ± 5.82 (n = 36)	52.88 ± 5.38 (n = 33)	35.55 ± 4.07 (n = 40)	53.86 ± 5.06 (n = 36)
Negative (n = 8)	61.21 ± 4.50	51.08 ± 4.43 (n = 12)	24.35 ± 3.80 (n = 20)	47.11 ± 5.88 (n = 19)	26.38 ± 3.53 (n = 21)	47.55 ± 4.57 (n = 20)

GBFT: Gallbladder filling time; GB t1/2: Gallbladder half emptying time; GBEF₃₀: Gallbladder ejection fraction at 30 min; GBEF₆₀: Gallbladder ejection fraction at 60 min; A: Experienced observer; B: Inexperienced observer.

RESULTS

¹⁴C-UBT was found as positive in 58 dyspeptic patients (35 male, 23 female, mean age of 41 years) and negative in 28 patients (9 male, 19 female, mean age of 45 years). In 74 cases, the sensitivity and specificity were determined as 88%-86% for Clo test and as 89%-80% for histologic evaluation respectively.

The parameters of GB function were not significantly different in *H pylori* positive and negative patients (*P* > 0.05) (Table 1). The GBFT of ¹⁴C-UBT positive patients (53.71 ± 3.49 min) did not differ significantly from that of ¹⁴C-UBT negative patients (61.21 ± 4.50 min) (*P* > 0.05). Minimum value of GBFT was 30 min and gallbladder filled at 30 min in 34 (39.5%) of the 86 cases. Two subjects who are one subject from *H pylori* positive group and the other one from negative group did not show gallbladder filling until the end of the acquisition. In 27 of 58 ¹⁴C-UBT positive patients (46.55%) and in 12 of 28 ¹⁴C-UBT negative patients (42.85%), GB emptying was observed (Figure 1). The GB t1/2 was 44.15 ± 4.38 min and 51.08 ± 4.43 min for ¹⁴C-UBT positive and negative patients, respectively and no significant difference was found between the two groups (*P* > 0.05). Mean GBEF values at 30 min (GBEF₃₀) and at 60 min (GBEF₆₀) obtained by the experienced observer (A) in ¹⁴C-UBT positive patients

Table 2 Correlation values of GBEF₃₀ and GBEF₆₀ between two observers in ¹⁴C-UBT positive and negative patients

	A	B	r value
¹⁴ C-UBT Positive			
GBEF ₃₀	36.58 ± 5.82	35.55 ± 4.07	0.78
GBEF ₆₀	52.88 ± 5.38	53.86 ± 5.06	0.94
¹⁴ C-UBT Negative			
GBEF ₃₀	24.35 ± 3.80	26.38 ± 3.53	0.88
GBEF ₆₀	47.11 ± 5.88	47.55 ± 4.57	0.88

GBEF₃₀: Gallbladder ejection fraction at 30 min; GBEF₆₀: Gallbladder ejection fraction at 60 min; A: Experienced observer; B: Inexperienced observer; r value: Correlation coefficient.

were 36.58% ± 5.82% and 52.88% ± 5.38%, respectively. In ¹⁴C-UBT negative patients GBEF₃₀ was 24.35% ± 3.80% and GBEF₆₀ was 47.11% ± 5.88%. Mean GBEF values at 30 min and at 60 min obtained by the inexperienced observer (B) in ¹⁴C-UBT positive patients were 35.55% ± 4.07% and 53.86% ± 5.06%, and in ¹⁴C-UBT negative patients were GBEF₃₀ 26.38% ± 3.53% and GBEF₆₀ 47.55% ± 4.57%, respectively. GBEF values did not differ significantly (*P* > 0.05). A highly significant positive correlation of corresponding values was found between the two observers (Table 2).

DISCUSSION

There is no previously published study regarding the direct relationship between the gallbladder motor functions and *H pylori* infection in dyspeptic patients. In the previous studies, the relationship between gastric and gallbladder emptying functions were reported. But, no definitive physiological data of the gallbladder kinetic parameters has been published yet^[20,25-29]. Marzio *et al.*^[25,26] showed that gastric emptying is strictly correlated with gallbladder emptying and refilling. It has been reported that impairment of gallbladder dynamic functions might be due to inflammation resulting from *H pylori* infection. In our dyspeptic patients, high incidence of *H pylori* infection (67%) and delayed GBFT support the hypothesis that this bacteria can cause dyspepsia^[2,7,15,16]. On the other hand, since *H pylori* was found as negative in 33% of our dyspepsia patients, it seemed unlikely that *H pylori* was the unique factor for dyspepsia. Abnormal bile composition may be responsible even if GB USG is normal. Further studies in patients with dyspepsia would be helpful in clarifying this issue.

In our study, each subject was studied with a standardized fatty meal releasing endogenous CCK as a stimulant for GB emptying. Krishnamurthy *et al.*^[30,31] reported that the GBFT and the GB latent period before the beginning of emptying were much longer, and GBEF values at 60 min were significantly lower obtained with fatty meal ingestion than with CCK injection. It probably resulted from the time taken for release of endogenous CCK. They suggested that acquisition has to last for at least 60 min. Dependent on these results, we acquired GB kinetic images up to 90 min and our results supported the reports of Krishnamurthy *et al.*^[30,31]. In all of our cases, GBFT and GB t1/2 time were increased while the mean GBEF value at 60 min was decreased. Interestingly, that GB functions impaired more prominently in *H pylori* negative patients was observed, however no statistically significant difference was detected between the two groups for each observer (Tables 1 and 2).

In conclusion, cholescintigraphy using ^{99m}Tc-Mebrofenin and a fatty meal ingestion is a well established and reliable noninvasive method for estimating gallbladder motor functions. Since we did not find any significant difference in gallbladder kinetic parameters between *H pylori* positive and negative patients with dyspepsia, *H pylori* did not seem to cause the abnormal gallbladder function (filling or emptying). Up to date, direct relationship between *H pylori* infection and gallbladder motor functions has not been reported. For that reason, we are not able to compare our results directly with any other published data, and further studies on this topic may help to clarify our findings.

COMMENTS

Background

The *H pylori* is an interesting cause of active chronic gastritis and duodenitis or even cancer worldwide. The presence of *H pylori* could also predispose to various disorders such as dental disease, atherosclerosis, coronary artery disease, slow coronary flow and cerebral infarction. Few reports have been published on the relationship between gallbladder emptying in patients with *H pylori* positive and negative idiopathic dyspepsia. Our aim was scintigraphic evaluation of gallbladder motor functions in *H pylori* positive and negative patients affected by dyspepsia.

Research frontiers

Higher incidence of *H pylori* infection in dyspeptic patients supports the idea that it can cause to the development of dyspepsia. In the previous studies on dyspeptic patients showed that a group of dyspeptic patients had a reduced gallbladder response to a liquid meal. However, no definitive data of the gallbladder kinetic parameters has been published in the previous studies.

Innovations and breakthroughs

¹⁴C-UBT is reliable noninvasive method for the diagnosis of *H pylori* infection. Up to date, direct relationship between *H pylori* infection and gallbladder motor functions has not been studied. We showed for the first time that the gallbladder motor functions such as filling time, ejection fraction and emptying time values were not affected from *H pylori* infection.

Applications

Our study was designed to analyse the scintigraphic gallbladder motor function parameters in *H pylori* positive and negative patients based on dyspeptic symptoms, ¹⁴C-UBT and upper gastrointestinal endoscopy procedure. The *H pylori* did not appear to cause the impairment in gallbladder function.

Terminology

¹⁴C-UBT: Urea breath test using ¹⁴C capsule is based on the principle that urease activity is present in the stomachs of individuals affected with *H pylori*; GBEF: Gallbladder ejection fraction parameter describes gallbladder emptying function; ^{99m}Tc-Mebrofenin: It is a radiopharmaceutical agent for hepatobiliary scintigraphy.

Peer review

This is a report designed to analyse the gallbladder motor function parameters in *H pylori* positive and negative patients with dyspepsia. This clinical study was well designed.

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Relevance of MUC1 mucin variable number of tandem repeats polymorphism in *H pylori* adhesion to gastric epithelial cells

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MUC1 VNTR domain. The adhesion is further dependent on bacterial pathogenicity and the gastric cell line. MUC1 mucin variability may contribute to determine *H pylori* colonization of the gastric mucosa.

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Key words: *H pylori*; MUC1; Variable number of tandem repeats; Polymorphism; Adhesion; Mucin; Gastric; Infection

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Abstract

AIM: To evaluate the influence of MUC1 mucin variable number of tandem repeats (VNTR) variability on *H pylori* adhesion to gastric cells.

METHODS: Enzyme linked immunosorbent assay (ELISA)-based adhesion assays were performed to measure the adhesion of different *H pylori* strains (HP26695 and HPTx30a) to gastric carcinoma cell lines (GP202 and MKN45) and GP202 clones expressing recombinant MUC1 with different VNTR lengths.

RESULTS: Evaluation of adhesion results shows that *H pylori* pathogenic strain HP26695 has a significantly higher ($P < 0.05$) adhesion to all the cell lines and clones tested, when compared to the non-pathogenic strain HPTx30a. Bacteria showed a significantly higher ($P < 0.05$) adhesion to the GP202 cell line, when compared to the MKN45 cell line. Furthermore, both strains showed a significantly higher ($P < 0.05$) adhesion to GP202 clones with larger MUC1 VNTR domains.

CONCLUSION: This work shows that MUC1 mucin variability conditions *H pylori* binding to gastric cells. The extent of bacterial adhesion depends on the size of the

INTRODUCTION

The Gram negative bacterium *H pylori* is involved in the pathogenesis of several gastrointestinal diseases, ultimately leading to gastric carcinoma^[1,2]. In the gastric mucosa, the majority of the bacteria is found within the mucus layer, but can be also attached to gastric epithelial cells^[3], a crucial step for the maintenance, spreading and severity of the infection. This attachment is mediated by the interaction of bacterial molecules, such as adhesins and LPS^[4], with gastric cell surface ligands such as glycolipids and glycoproteins. MUC1 is a membrane glycoprotein that protects epithelial surfaces and has been recently identified as an *H pylori* binding target^[5,6]. Extracellular MUC1 variable number of tandem repeats (VNTR) domain is highly glycosylated^[7], presenting carbohydrate structures (e.g. Lewis b carbohydrate antigen) involved in the binding of *H pylori* through its adhesins BabA and SabA^[8,9]. Furthermore this repetitive region shows extensive allelic variation ranging from 25-125 repeat units^[12]. The relevance of MUC1 VNTR variability for *H pylori* adhesion to gastric cells remains to be clarified.

In this work we tested the hypothesis that MUC1 VNTR polymorphism affects the *H pylori* adhesion to gastric cells and thus plays an important role in the colonization

of gastric mucosa. We used *H. pylori* strains with different pathogenicity (strain HP26695 and strain HPTx30a) co-cultured with gastric cell lines GP202 and MKN45, and GP202 clones expressing recombinant MUC1 with different VNTR lengths. Adhesion was evaluated by an enzyme linked immunosorbent assay (ELISA)-based adhesion assay.

The results showed that MUC1 VNTR polymorphism influences the binding of *H. pylori* to gastric cells. Furthermore, higher adhesion was observed in co-cultures with the pathogenic strain (HP26695) when compared to the non-pathogenic strain (HPTx30a) and GP202 cell line when compared to the MKN45 cell line. This work contributes to the understanding of the interplay between host and bacterial factors in *H. pylori* infection pathogenesis.

MATERIALS AND METHODS

Cell lines

We used two gastric carcinoma cell lines: GP202, previously established in our laboratory^[13] from a signet ring cell gastric carcinoma that constitutively expresses MUC1 and MKN45 (Japan Health Sciences Foundation).

GP202 clones expressing recombinant MUC1 with different VNTR lengths^[14] were previously established by stable transfection with an eukaryotic expression vector pHb-APr1-neo containing subcloned epitope-tagged MUC1 (FLAG-MUC1) cDNAs with different number of TR units (0, 3, 9 and 42 repeats, respectively GP202-dTR, GP202-3TR, GP202-9TR and GP202-42TR)^[15]. GP202-Neo was obtained by transfection with empty vector.

The parental cell lines and transfectants were cultured in 150 cm² flasks at 37°C in a humidified 5% CO₂ incubator and maintained in RPMI 1640 medium (with Glutamax and 25 mmol/L Hepes) supplemented with 10% fetal bovine serum and 50 µg/mL gentamicin. Media was changed every 3 d to 4 d, and the cells were passaged when they reached 80% to 90% confluence using 0.05% trypsin-0.53 mmol/L ethylenediamine tetra-acetic acid in Hank's balanced salt solution. Cell culture reagents were obtained from Invitrogen (Carlsbad, CA, USA).

H. pylori strains

Two *H. pylori* strains were used in this study: the pathogenic strain HP26695 (*vacA* s1/m1, *cag* PAI+, ATCC 700392) and the non-pathogenic strain HPTx30a (*vacA* s2/m2, *cag* PAI-, ATCC 51932). Bacteria were grown on Trypticase soy agar with 5% sheep blood (BioMérieux) at 37°C in microaerobic conditions.

ELISA assay

Quantitative evaluation of *H. pylori* adhesion to gastric cells was performed by ELISA, as previously described^[16], with some modifications. Briefly, cells were cultured in 96 well plates and allowed to form confluent monolayers. Cells were washed and *H. pylori* suspension was added in a 200:1 bacteria to cell ratio (MOI) and incubated for 60 min. Cells were washed and fixed at 4°C with 8% paraformaldehyde for 60 min. Endogenous peroxidase was inactivated by addition of 1% H₂O₂ in methanol. After washing with PBS, anti-*H. pylori* monoclonal antibody MAB922 (Chemicon, USA) was added overnight, 4°C, followed

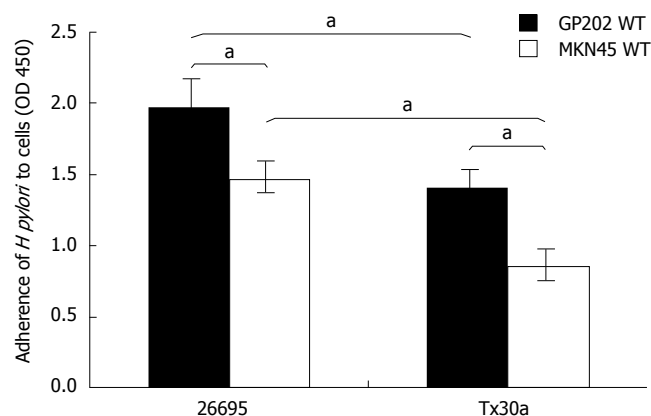


Figure 1 Adhesion of HP26695 and HPTx30a *H. pylori* strains to GP202 and MKN45 gastric cell lines. ^a*P* < 0.05.

by addition of peroxidase-conjugated goat anti-mouse immunoglobulins (Santa Cruz Biotechnology) 30 min, RT. Tetramethylbenzidine (TMB) (Sigma, USA) was added and reaction stopped with 1 mol/L HCl. Plates were read in a 680 ELISA microplate reader (Bio-Rad, USA) at 450 nm. OD values were used as the index of the number of *H. pylori* adhering to cells^[16]. Two sets of triplicates were made for each assay.

Statistical analysis

Statistical analysis was performed using the Mann-Whitney test, StatView Software version 5.0 (SAS Institute). A *P* value of less than 0.05 was accepted as statistically significant.

RESULTS

Evaluation of *H. pylori* adhesion shows that pathogenic strain HP26695 has significantly (*P* < 0.05) higher adhesion values for both GP202 and MKN45 cell lines (1.97 ± 0.10 and 1.47 ± 0.06) when compared with the non-pathogenic strain HPTx30a (1.40 ± 0.15 and 0.85 ± 0.15 ; Figure 1). This statistically significant association between pathogenicity and higher adhesion (strain HP26695 *vs* HPTx30a) is also observed for the GP202 MUC1 recombinant clones (GP202-Neo 1.1 ± 0.10 *vs* 0.72 ± 0.06 ; GP202-dTR 1.32 ± 0.09 *vs* 1.0 ± 0.10 ; GP202-3TR 1.45 ± 0.08 *vs* 1.18 ± 0.05 ; GP202-9TR 2.2 ± 0.12 *vs* 1.96 ± 0.12 ; and GP202-42TR 2.3 ± 0.07 *vs* 1.89 ± 0.11 ; Figure 2). Furthermore, GP202 cell line shows higher adhesion levels than MKN45 cell line for both bacteria strains (HP26695 strain 1.97 ± 0.10 *vs* 1.47 ± 0.06 ; HPTx30a strain 1.40 ± 0.15 and 0.85 ± 0.15 ; Figure 1).

Adhesion of both *H. pylori* strains (HP26695 and HPTx30a) is significantly higher in all the GP202-MUC1 transfectants over-expressing MUC1 (GP202-dTR 1.32 ± 0.09 and 1.0 ± 0.10 ; GP202-3TR 1.45 ± 0.08 and 1.18 ± 0.05 ; GP202-9TR 2.2 ± 0.12 and 1.96 ± 0.12 ; GP202-42TR 2.3 ± 0.07 and 1.89 ± 0.11) when compared with the control, GP202 Neo (1.1 ± 0.10 and 0.72 ± 0.06 , Figure 2). There is also an association between the increased number of Tandem Repeats (GP202-9TR and GP202-42TR) and the increased adhesion, for both strains (Figure 2).

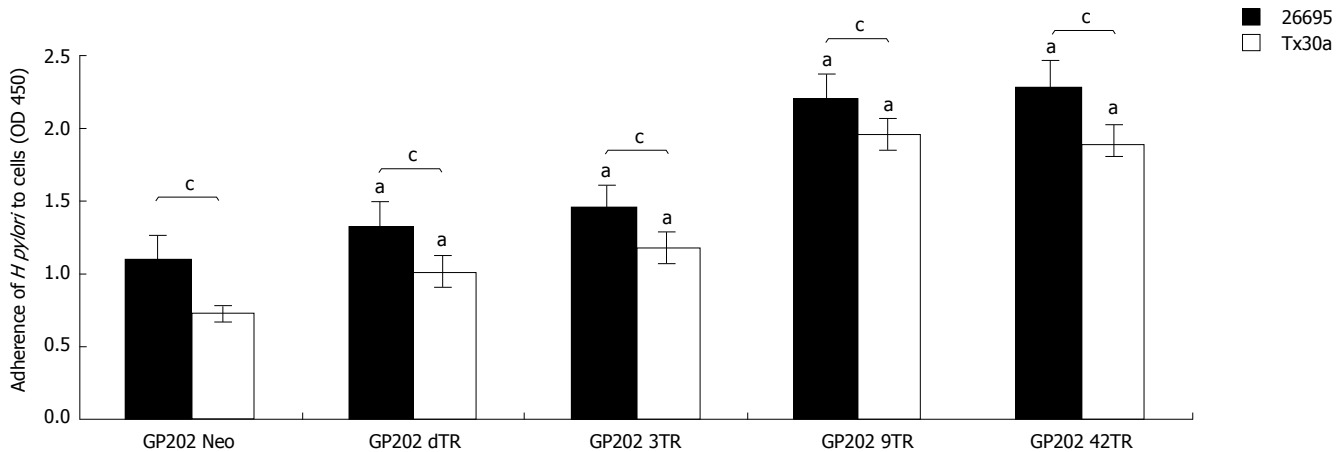


Figure 2 Adhesion of HP26695 and HPTx30a *H pylori* strains to GP202 transfectants GP202-Neo, GP202-dTR, GP202-3TR, GP202-9TR and GP202-42TR. * $P < 0.05$, compared to the control (GP202 Neo) and ^b $P < 0.05$.

DISCUSSION

Epidemiological studies and animal models have shown that *H pylori* chronic infection is associated with several gastric pathologies, ranging from asymptomatic gastritis to gastric adenocarcinoma and MALT lymphoma^[1,2]. The different consequences of the infection suggest that several factors from the host and the bacteria are involved in the bacteria-host interactions, being the pathogenic potential dependent upon the molecular context of the colonization of gastric mucosa. To date several factors involved in the *H pylori* infection have already been identified (e.g. bacterial adhesins, host mucins and pro-inflammatory cytokines) however the complete mechanism remains to be clarified^[17-19].

Adhesion of *H pylori* to gastric mucosa is a fundamental step for epithelium colonization. Different adhesion mechanisms, commonly targeting carbohydrate structures present on gastric cells surface, have been identified^[4] with *H pylori* ligands including, among others, blood group antigens on mucins and glycolipids^[8-11,20-26].

The best-characterized *H pylori* adhesin is BabA, that mediates a strong adhesion between the bacteria and Le^b blood group antigen expressed on the surface of epithelial cells^[8,27]. This work showed that adhesion is a relevant feature of *H pylori* pathogenicity potential, with significantly higher adhesion levels observed for the HP26695 (pathogenic strain) when compared to the HPTx30a (non-pathogenic strain) in both cell lines. Considering that both strains don't express BabA adhesin^[28], the observed differences can not be explained through the BabA binding model, what suggests that other bacterial molecules are involved in the adhesion process.

Another important observation is that there is a higher adhesion of HP26695 and HPTx30a strains to GP202 cell line when compared with MKN45 cell line. This reflects different expression levels and availability of ligands at the cells surface. Previous characterization of mucins and carbohydrate expression on GP202 and MKN45 cell lines showed that Le^b has a significantly higher expression in GP202 cell line^[29]. Still, this difference might not be relevant since BabA is not present in both bacterial strains^[28]. In addition, the MUC1 expression is

identical for both cell lines^[29] and therefore can not be held responsible for the observed differences. GP202 has a higher expression of other carbohydrate antigens (Le^a and Le^y)^[29,30] compared to MKN45, that might be involved in *H pylori* binding interactions. Moreover, additional ligands/interactions that are not yet explored may also exist that can explain this difference in adhesion levels between cell lines.

In order to study the influence of MUC1 VNTR variability in *H pylori* binding, we used GP202, the cell line that showed higher bacteria adhesion and we analyzed GP202 transfected clones expressing recombinant MUC1 with a different number of repeats. These clones overexpress similar levels of recombinant MUC1^[14]. We observed that MUC1 VNTR polymorphism has influence in the extent of *H pylori* binding to gastric cells, with the higher adhesion levels observed in clones with larger VNTR regions. This may be due to the fact that MUC1 with larger Tandem Repeat regions contains more potential glycan receptors, thus potentially providing more bacterial binding sites. Moreover, we have previously shown that differences in VNTR length lead to glycosylation changes in the MUC1 Tandem Repeat^[14], which may also contribute to the altered adhesion observed. Detailed evaluation of the results showed a small increase between the adhesion of GP202-NEO (control) and GP202-dTR that may be explained by the overexpression of MUC1 in recombinant clone GP202-dTR^[14] and by the potential presence of O-glycosylated binding sites outside the VNTR region. No significant difference was observed between the adhesion of the bacteria to GP202-9TR and to GP202-42TR clones. We have previously observed the overexpression of MUC1 underglycosylated forms in GP202-42TR^[14], which might explain why the adhesion levels are not proportional to VNTR size.

All these observations are important for understanding the bacterial and host molecular context of the colonization of gastric mucosa. Identification of a pathogenesis background, based upon host susceptibility traits like MUC1 VNTR polymorphism, will help to identify candidates more prone to bacterial colonization and patients more resilient to eradication strategies.

COMMENTS

Background

More than half of the world population is persistently infected by *H pylori*. Adhesion of the bacteria to the gastric mucosa is essential for attachment and infection. Therefore it is important to know host and bacterial factors that condition the adhesion.

Innovations and breakthroughs

The study of host factors that influence the binding of *H pylori* to gastric cells may help to identify candidates more prone to bacterial colonization and patients more resilient to eradication strategies.

Applications

These findings may help to develop screening methods to identify candidates more prone to bacterial colonization and to develop more efficient eradication strategies, as well as to develop strategies to prevent or minimize *H pylori* binding to the gastric mucosa.

Peer review

This is a good study designed to elucidate that MUC1 VNTR polymorphism affects *H pylori* adhesion to gastric cells. The results are informative and potentially helpful for prevention of *H pylori* binding to the gastric mucosa.

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Fatty liver disease in severe obese patients: Diagnostic value of abdominal ultrasound

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US was associated to advanced grades of steatosis on histology ($P = 0.016$).

CONCLUSION: Preoperative abdominal US in our series has not shown to be an accurate method for the diagnosis of hepatic steatosis in severe obese patients. Until another non-invasive method demonstrates better sensitivity and specificity values, histological evaluation may be recommended to these patients undergoing bariatric surgery.

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Key words: Bariatric surgery; Obesity; Hepatic steatosis; Abdominal ultrasound diagnosis; Fatty liver

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Abstract

AIM: To evaluate the sensitivity and specificity of abdominal ultrasound (US) for the diagnosis of hepatic steatosis in severe obese subjects and its relation to histological grade of steatosis.

METHODS: A consecutive series of obese patients, who underwent bariatric surgery from October 2004 to May 2005, was selected. Ultrasonography was performed in all patients as part of routine preoperative time and an intraoperative wedge biopsy was obtained at the beginning of the bariatric surgery. The US and histological findings of steatosis were compared, considering histology as the gold standard.

RESULTS: The study included 105 patients. The mean age was 37.2 ± 10.6 years and 75.2% were female. The histological prevalence of steatosis was 89.5%. The sensitivity and specificity of US in the diagnosis of hepatic steatosis were, respectively, 64.9% (95% CI: 54.9-74.3) and 90.9% (95% CI: 57.1-99.5). The positive predictive value and negative predictive value were, respectively, 98.4% (95% CI: 90.2-99.9) and 23.3% (95% CI: 12.3-39.0). The presence of steatosis on

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) has been recognized as an important and common clinical entity, affecting approximately 20% of the general population^[1]. The prevalence of NAFLD in obese people has been estimated in 60%-95% and, currently, NAFLD has been suggested to be the liver component of the metabolic syndrome^[2,3]. It has a large spectrum, ranging from simple hepatic steatosis to steatohepatitis (NASH) and cirrhosis.

Liver biopsy and histological evaluation have been considered the better methods for the diagnosis of steatosis and to establish the prognosis of NAFLD. However, there are controversies about the indication of biopsy in clinical practice, due the lack of an effective medical therapy for NAFLD and the risks associated with this procedure^[4].

Nevertheless, various imaging modalities have been used to diagnose the presence of fat in the liver, as ultrasonography, computerized tomography scan and magnetic resonance imaging. Abdominal ultrasound (US), the

cheapest method, has been the most common modality used in clinical practice. Some parameters allow the diagnosis of fatty liver disease with a sensibility of 83% and a specificity of 100%: a diffuse hyperechoic echotexture; deep attenuation; increased liver echotexture compared with the kidney; and vascular blurring^[5]. However, lower sensitivity values of US have been demonstrated among severe obese people^[6]. The present study aimed to evaluate the reliability of abdominal US when it is compared to histology to diagnose hepatic steatosis in these patients and its relation to histological grades of steatosis.

MATERIALS AND METHODS

Study group selection

A consecutive series of obese patients who underwent bariatric surgery from October 2004 to May 2005 was selected. The eligible criteria for inclusion were: age above 18 years, preoperative abdominal US, and liver biopsy during the surgery and signer agreement to participate the study. All patients had body mass index above 40 kg/m², or 35 kg/m² associated to others conditions (hypertension, diabetes, dyslipidemia or sleep apnea)^[7]. Patients with alcohol intake above 20 g/d or those who had other chronic liver diseases (HBV or HCV infection, hemochromatosis, autoimmune hepatitis, Wilson's disease, primary biliary cirrhosis, α -1 antitrypsin-deficiency) were excluded. The same surgeon team made all surgeries. Abdominal US was performed in all patients as part of routine preoperative time and different radiologists carried them out.

This study was performed in accordance with a protocol approved by Ethics Committee for Medical Research of Gonçalo Muniz Research Center. All included patients have consented to their participation in this study.

Clinical evaluation

The parameters studied included: age, gender, height, weight, waist circumference, history of hypertension, dyslipidemia, diabetes and drugs use. Laboratory evaluation included: hemoglobin, hematocrite, leucocytes, prothrombin time, ASL, ALT, total bilirubin, albumin, total cholesterol, HDL-cholesterol, triglycerides, fasting plasma glucose and insulin. Abdominal US exam was performed in all patients. Insulin resistance was calculated using Homeostasis Model Assessment Index (HOMA-IR). Patients were categorized as insulin resistant if the HOMA-IR value was equal or greater than 3.0, as previously described^[8]. Ultrasonographic definition of steatosis was based on diagnosis criteria usually used in clinical practice, as mentioned above^[5].

Liver biopsy and histological analysis

An intraoperative wedge biopsy was obtained at the beginning of the surgery and all samples were processed and examined by a single pathologist, using hematoxylin-eosin stain. Hepatic steatosis in the biopsy specimens, if present, was graded according the number of involved hepatocytes: Grade I (steatosis in 5%-25% of hepatocytes); Grade II (steatosis in 25%-50% of hepatocytes); Grade III (steatosis in 50%-75% of hepatocytes); Grade IV (steatosis in more than 75% of hepatocytes).

Statistical analysis

Data were processed and analyzed using the Statistical Package for Social Science program, version 9.0 (SPSS Inc. Chicago, Illinois, USA). Descriptive statistics of the included variables has been carried out. The US and histological findings of steatosis were compared, considering histology as the gold standard. Subsequently, the sensibility, specificity, positive predictive value and negative predictive value for the US in the diagnosis of hepatic steatosis and theirs 95% confidence intervals were calculated using EPI INFO v6.0 (CDC, USA). A Chi-square test was used to compare categorical variables. All statistical methods were two-tailed and the statistical significance was obtained when $P < 0.05$.

RESULTS

Among the 123 severe obese patients with histological and abdominal US evaluation, 105 were finally included. Seventeen patients were excluded because they had a history of alcohol intake above 20 g/d, and one by hepatitis B infection (HBsAg positive).

Demographic and clinical profiles of these 105 individuals are shown in Table 1. The US examination was normal in 38 (36.2%) cases. Steatosis on US was described in 62 (59.0%) cases. Others imaging findings were: hepatomegaly in 8 (7.6%); colelithiasis in 14 (13.3%); and renal cyst in 3 (2.9%) cases.

The histological prevalence of steatosis was 89.5%. The sensitivity and specificity of abdominal US for the diagnosis of hepatic steatosis were, respectively, 64.9% (95% CI: 54.9-74.3) and 90.9% (95% CI: 57.1-99.5). The positive and negative predictive values were, respectively, 98.4% (95% CI: 90.2-99.9) and 23.3% (95% CI: 12.3-39.0). A false positive rate was found in 9.1% (95% CI: 0.5-37.3) and a false negative rate in 35.1% (95% CI: 26.0-45.2).

Table 2 shows the influence of body mass index on accuracy of abdominal US in the diagnosis of hepatic steatosis. The prevalence of steatosis in patients with body mass index between 35.0 kg/m² and 39.9 kg/m² and in patients with body mass index above 40 kg/m² was 83.3% and 91.3%, respectively.

All individuals were separated into two groups, according to the median of waist circumference (below and above the median value) and the sensibility, specificity, positive predictive value and negative value were analyzed in each group. These results are demonstrated in Table 3. The prevalence of steatosis in patients below and above the median value for waist circumference was 81.1% and 94.6%, respectively.

The presence of steatosis on US was associated with advanced grades of steatosis in the biopsy specimens ($P = 0.016$), as shown in Table 4.

DISCUSSION

Abdominal US has been largely used in clinical practice and in protocols of investigation of patients with NAFLD because it is a cheap and a safe method. As a screening test, its major requirement is a high degree of sensitivity and specificity. In non-obese patients the values of sensitivity

Table 1 Demographic and clinical characteristics of severe obese patients who underwent to bariatric surgery

Characteristics	Value
Female gender - <i>n</i> (%)	79 (75.2)
Age, in yr - mean \pm SD	37.2 \pm 10.6
BMI, in kg/m ² - mean \pm SD	43.8 \pm 5.2
Elevated waist circumference - <i>n</i> (%)	105 (100)
Hypertension - <i>n</i> (%)	55 (52.4)
Diabetes - <i>n</i> (%)	10 (9.5)
Dyslipidemia - <i>n</i> (%)	75 (71.4)
Exposure to chemicals - <i>n</i> (%)	10 (9.5)
Elevated transaminases - <i>n</i> (%)	30 (28.6)
HDL cholesterol - mean \pm SD	46.5 \pm 5.2
Triglyceride - mean \pm SD	157.3 \pm 82.8
Fasting plasma glucose level - mean \pm SD	102.6 \pm 40.8
Insulin resistance - <i>n</i> (%)	52 (49.5)

BMI: Body mass index.

Table 2 Levels of sensibility, specificity, PPV and NPV for the ultrasound in the diagnosis of hepatic steatosis by body mass index (BMI) values

Variables	35.0 kg/m ² \leq BMI \leq 40 kg/m ²		BMI \geq 40 kg/m ²	
	Value (%)	95% CI	Value (%)	95% CI
Sensitivity	65	40.9-83.7	64.4	52.2-75.0
Specificity	75	21.9-98.7	100	56.1-100
PPV	92.9	64.2-99.6	100	90.6-100
NPV	30	8.1-64.6	21.2	9.6-39.4

PPV: Positive predictive value; NPV: Negative predictive value; 95% CI: Confidence interval of 95%.

and specificity of US range from 83% to 94%, and 84% to 100% respectively^[5,9].

The present study with severely obese patients evaluated the sensitivity and specificity of abdominal US for the diagnosis of hepatic steatosis and its relation to histology. The results showed a low performance of US to diagnosis steatosis, however similar results have been demonstrated in patients with body mass index ranging from 35.0 kg/m² to 82.2 kg/m², where the frequency of steatosis was 91.4%^[6]. In this case, sensitivity and specificity of US in the diagnosis of steatosis was 49.1% and 75%, respectively^[6]. These values are also demonstrated (sensitivity: 43%; specificity: 79%) for the diagnosis hepatic steatosis in patients infected with hepatitis C virus^[10].

Several hypotheses may explain this low performance of US in severe obese people. The diagnosis made by different radiologists may introduce variability in interpretation of images. This could be related to the experience of each radiologist and to the lack of clear standards for the diagnosis of hepatic steatosis^[11]. The second hypothesis is related to the adipose tissue thickness that may cause technical difficulties for the performance of this exam. The thick layers of subcutaneous fat in obese people may mislead the examiner's judgment of liver echogenicity, as cited in visualizing of the abdominal aorta^[12] and renal carcinoma^[13]. The image quality rate also has been discussed and different results have as been found. The analysis of 140 patients, who underwent

Table 3 Levels of sensibility, specificity, PPV and NPV for the ultrasound in the diagnosis of hepatic steatosis by waist circumference (WC) in severe obese patients

Variables	WC \leq 116.9 kg/m ²		WC \geq 117.0 kg/m ²	
	Value (%)	95% CI	Value (%)	95% CI
Sensitivity	56.7	37.7-74.0	80	62.5-90.9
Specificity	85.7	42.0-99.2	100	19.8-100
PPV	94.4	70.6-99.7	100	85.0-100
NPV	31.6	13.6-56.5	22.2	3.9-59.8

PPV: Positive predictive value; NPV: Negative predictive value; 95% CI: Confidence interval of 95%.

Table 4 Ultrasound evaluation and grades of steatosis among severe obese patients with steatosis on biopsy (%)

Imaging profile	Grade of steatosis on histological evaluation	
	Grade I / II	Grade III / IV
Ultrasound without steatosis	97.00	3.00
Ultrasound with steatosis	77.00	23.00

P = 0.016.

abdominal US, showed that obesity was also associated with a poor sonographic image^[14]. However, another study did not find the same results^[15]. Finally, the majority of screening values described in literature were calculated in patients with suspected liver disease. It is more appropriate to use groups of patients resembling those that have been investigated in clinical practice.

This investigation found a higher accuracy of US for the diagnosis of hepatic steatosis in patients with central obesity or more elevated waist circumference. This could be explained by the association between steatosis on ultrasonographic evaluation and histological evidence of steatosis in those patients, as previously showed^[9,16]. In a multivariate model involving patients with hepatitis C, Hepburn found that the only statistically significant factor associated with steatosis on US was histological grade, with an odds-ratio of 3.6^[10]. In addition, a better performance of ultrasound associated to a more elevated prevalence of NAFLD was also described in obese people undergoing bariatric surgery^[6], obtaining results as high as those found in non-obese people^[5,9].

The prevalence of steatosis among the patients in this study was elevated (89.5%) and the central obesity was frequent, as previously described studies^[6,17,18]. Moreover, the prevalence of NAFLD also has been correlated to body mass index. These results are relevant when we considered that obesity is associated to increased visceral adiposity, free fatty acids and hyperinsulinemia, which are involved in the pathogenesis of NAFLD^[19,20].

In conclusion, the results suggest that abdominal US may not be considered an accurate method for the diagnosis of hepatic steatosis in severe obese patients. The liver biopsy and histological evaluation should be recommended to these patients undergoing bariatric surgery, until other non-invasive method demonstrates better sensitivity and specificity values.

COMMENTS

Background

Non-alcoholic fatty liver disease (NAFLD) is a common entity among severe obese patients. Although liver biopsy was the best method for its diagnosis, various imaging modalities have been used to diagnose the presence of fat in the liver, and abdominal ultrasound (US) is the most used in clinical practice. Thus, it is important to elucidate if the abdominal US is a good method to diagnose hepatic steatosis in these patients.

Research frontiers

This study reported the findings of 105 patients with a histological prevalence of steatosis of 89.5%. Low sensitivity and negative predictive rates of US in the diagnosis of hepatic steatosis were described and a better performance of US was associated to advanced grades of steatosis on histology. More accurate methods may change this data.

Innovations and breakthroughs

Although the reliability of abdominal US for diagnose hepatic steatosis was previously reported, the current study showed different values of specificity and sensibility on severe obese patients by body mass index and waist circumference status and the relationship between grade of steatosis on histology and its presence on US evaluation.

Applications

Abdominal US results should be carefully analysed in obese patients. Because of the high prevalence of non-alcoholic fatty liver disease, a more accurate method for its diagnosis, as liver biopsy, is recommended in patients undergoing bariatric surgery.

Terminology

NAFLD means non-alcoholic fatty liver disease; NASH is named as non-alcoholic steatohepatitis; HOMA-IR is homeostasis model assessment index - insulin resistance.

Peer review

The authors evaluate the use of US in severely obese patients in order to diagnose hepatic steatosis. They compare histological findings, which they consider to be the golden standard, with US data of the same patients. They observe 123 cases and conclude that US may not be considered an accurate method for the diagnosis of steatosis in these patients. The goals of the study, the materials and methods, results and other parts of the manuscript are well formulated and explain the study in well chosen terms. As a histologist, it is nice to hear that histology is the method of choice for this diagnosis, in spite of the fact that US based diagnosis would have been a very direct, non-invasive and cheap method to use.

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Gastric motor effects of ghrelin and growth hormone releasing peptide 6 in diabetic mice with gastroparesis

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Abstract

AIM: To investigate the potential therapeutic significance of ghrelin and growth hormone releasing peptide 6 (GHRP-6) in diabetic mice with gastric motility disorders.

METHODS: A diabetic mouse model was established by intraperitoneal (*ip*) injection of alloxan. Diabetic mice were injected *ip* with ghrelin or GHRP-6 (20-200 μ g/kg), and the effects on gastric emptying were measured after intragastric application of phenol red. The effect of atropine, N^G-nitro-L-arginine methyl ester hydrochloride (L-NAME) or D-Lys³-GHRP-6 (a growth hormone secretagogue receptor (GHS-R) antagonist) on the gastropromotric effect of ghrelin or GHRP-6 (100 μ g/kg) was also investigated. The effects of ghrelin or GHRP-6 (0.01-10 μ mol/L) on spontaneous or carbachol-induced contractile amplitude were also investigated *in vitro*, in gastric fundic circular strips taken from diabetic mice. The presence of growth hormone secretagogue receptor 1a transcripts in the fundic strips of diabetic mice was detected by reverse transcriptase polymerase chain reaction (RT-PCR).

RESULTS: We established a diabetic mouse model with delayed gastric emptying. Ghrelin and GHRP-6 accelerated gastric emptying in diabetic mice with gastroparesis. In the presence of atropine or L-NAME, which delayed gastric emptying, ghrelin and GHRP-6 (100 μ g/kg) failed to accelerate gastric emptying. D-Lys³-GHRP-6 also delayed gastric emptying induced by the GHS-R agonist. Ghrelin and GHRP-6 increased the carbachol-induced contractile amplitude in gastric fundic

strips taken from diabetic mice. RT-PCR confirmed the presence of *GHS-R* mRNA in the strip preparations.

CONCLUSION: Ghrelin and GHRP-6 increase gastric emptying in diabetic mice with gastroparesis, perhaps by activating peripheral cholinergic pathways in the enteric nervous system.

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Key words: Gastric emptying; Ghrelin; Growth hormone releasing peptide 6; Growth hormone secretagogue receptor; Diabetic mice

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INTRODUCTION

Delayed gastric emptying occurs in more than 50% of patients with chronic diabetes mellitus (DM) and is always associated with impaired quality of life and diabetic control. While this delay is not always clinically apparent, the range of gastrointestinal symptoms may include nausea, vomiting, regurgitation, fullness, and bloating^[1]. Diabetic patients with poor gastric emptying have a number of possible metabolic consequences, including poor glycemic control, increased risk of postprandial hypoglycemia and variable drug absorption. At its worst, gastroparesis can lead to intractable vomiting and an inability to feed, and carries a poor prognosis^[2].

Present management of diabetic gastroparesis involves empirical use of prokinetic drugs such as domperidone, metoclopramide, cisapride^[2,3] and erythromycin^[4]. The effects of these drugs, however, are unpredictable. One possible explanation for this lack of sustained response to treatment is that gastroparesis may be originally associated with progressive autonomic neuropathy^[5,6].

Ghrelin, a 28-amino acid peptide with an octanoyl moiety at Ser³, was discovered in 1999 as the endogenous

ligand for the growth hormone secretagogue receptor (GHS-R), now often referred to as the ghrelin receptor^[7]. The ghrelin receptor, originally called GHS-R1a, has also been called GRLN receptor since the discovery of ghrelin^[8]. This receptor was first characterized, cloned and identified as the receptor for a family of synthetic ligands known as growth hormone secretagogues, which stimulate the release of growth hormone (GH)^[9]. Ghrelin and its receptor have been localized to the gastrointestinal tracts of many mammalian species, including the mouse, rat and humans^[7,10-13]. In rats^[14], mice^[15,16] and dogs^[17], ghrelin has been found to increase gastric emptying, and the site of action may involve the enteric nervous system. In rats and mice, a gastropromotile-like activity of ghrelin may be observed *in vitro* as an increase in neuronally mediated contractions evoked by electrical field stimulation (EFS)^[11,14]. Growth hormone-releasing peptide-6 (GHRP-6) is a synthetic peptide that causes release of GH, similar to the effect of ghrelin, but through an as yet unknown mechanism. Diabetic gastroparesis is a disabling condition with no consistently effective treatment; however, the effect of ghrelin and its synthetic peptide GHRP-6 on diabetic mice with gastroparesis has not been reported. Therefore, we investigated the potential therapeutic significance of ghrelin and GHRP-6 in diabetic mice with gastric motility disorders.

MATERIALS AND METHODS

Chemicals

Rat ghrelin, GHRP-6 and D-Lys³-GHRP-6 were obtained from Tocris Cookson (Bristol, UK). N^G-nitro-L-arginine methyl ester hydrochloride (L-NAME) was purchased from Bachem (Bubendorf, Switzerland). Atropine sulfate, phenol red and alloxan were obtained from Sigma (St Louis, Missouri, USA).

Preparation of experimental animals

C57 mice weighing 18-22 g were obtained from the experimental Animal Center of the Shanghai Academia Sinica. All procedures were approved by the Medical Ethics Committee of Shanghai Jiaotong University. Mice were housed in stainless steel cages at a controlled temperature (22 ± 2°C) and 60%-65% relative humidity with a normal 12 h light/dark cycle. Six mice were randomly selected as normal controls, and the rest were fed a high-fat diet. After exposure to the high-fat diet for 3 wk, the mice were fasted overnight with free access to water, and injected intraperitoneally (*ip*) with alloxan (0.2 g/kg body weight) dissolved in sterile normal saline solution. Seventy-two h later, the fasting blood glucose levels of the mice were determined using the glucose oxidase method with a Glucose Analyzer. Mice with a blood glucose level greater than 11.1 mmol/L were defined as diabetic (DM) mice. DM mice continued to feed without control of blood glucose for 4 wk; then, the mice that were defined to be DM mice with gastroparesis, as confirmed by subsequent tests, were used for further investigations.

Studies of gastric emptying *in vivo*

Mice were allowed free access to water 12 h before the

experiment. DM mice were injected with either ghrelin (0, 20, 50, 100, or 200 µg/kg; *ip*) or GHRP-6 (0, 20, 50, 100, or 200 µg/kg; *ip*) in a random order. Modulation of the effects of the growth hormone secretagogue receptor (GHS-R) agonists by pharmacological blockers was tested by *ip* administration of atropine (1 mg/kg), L-NAME (50 mg/kg) or D-Lys³-GHRP-6 (5 µmol/kg) 15 min before administration of the GHS-R agonist (ghrelin 100 µg/kg or GHRP-6 100 µg/kg). Each drug treatment group consisted of at least six DM mice. An additional group consisting of at least 6 DM mice were injected with saline as normal controls.

Immediately after the injection of the drug, 5 mg/kg body weight phenol red test meal (0.5 g/L in 0.9% NaCl with 1.5% methylcellulose) was administered intragastrically with an orogastric canula. The mice were sacrificed 20 min later. The stomach was clamped with a string above the lower esophageal sphincter and a string beneath the pylorus to prevent leakage of phenol red. The stomach was cut just beneath the strings and was frozen at -70°C until measurement of gastric emptying. Gastric emptying was determined spectrophotometrically using a previously described method^[18,19]. The stomach of each mouse was cut just above the lower esophageal sphincter and the pyloric sphincter. Phenol red remained largely in the lumen of the stomach, although some was trapped in the mucus layer of the stomach, and a very small amount of phenol red was reabsorbed in the mucosa after 20 min. The stomach and its contents were submerged in 5 mL of 0.1 mol/L NaOH. The stomach was minced, and these samples contained the total amount of phenol red present in the stomach. The samples were further diluted in 10 mL 0.1 mol/L NaOH and left at room temperature for 1 h. Five mL of the supernatant was then centrifuged at 800 × g for 20 min. The absorbance was read at a wavelength of 546 nm with a spectrophotometer (Shanghai Yixian Company, China), and the phenol red content present in the stomach was calculated. The percentage of gastric emptying of the mice was calculated as (infusion-remained/infusion) × 100%.

Contractility measurements *in vitro*

DM mice were sacrificed by cervical dislocation, and the stomach was removed and rinsed with ice cold saline. Circular muscle strips, freed from mucosa (length 8-10 mm, width 0.2 mm) were cut from the fundus and suspended vertically in an organ bath filled with Krebs solution (120.9 mmol/L NaCl, 2.0 mmol/L NaH₂PO₄, 15.5 mmol/L NaHCO₃, 5.9 mmol/L KCl, 1.25 mmol/L CaCl₂, 1.2 mmol/L MgCl₂, and 11.5 mmol/L glucose) warmed at 37°C and gassed with 95% O₂/5% CO₂. One end of the strip was fixed to a hook on the bottom of the chamber while the other end was connected by a thread to an external isometric force transducer (BK Company, USA) at the top. After 1 h of equilibration at optimal stretch (0.75 g), the reproducibility of the contractile response to carbachol (0.1 µmol/L) was assessed. Mechanical responses in the smooth muscle strips were measured using an isometric force transducer and stored on a computer for analysis using the SMUP-E biological signal processing system (Chengdu Equipment Factory, China). To investigate the modification

of neuro-effector transmission by GHS-R agonists, the response was studied in the presence and absence of carbachol (0.1 $\mu\text{mol/L}$), which, when used, was added to the tissue bath 0.5 min before application of the GHS-R agonists. The effect of GHS-R agonists on spontaneous or carbachol (0.1 $\mu\text{mol/L}$)-induced contractile activity in DM mouse fundic muscle strips was studied by measuring the mean contractile amplitude of the muscle strips.

Measurement of the growth hormone secretagogue receptor by RT-PCR

Total RNA was prepared from DM mouse fundic muscle strips using Trizol reagent (Invitrogen, Carlsbad, CA). Single-stranded cDNA was synthesized using an oligo (dT) anchor primer and SuperscriptTM II RNase H⁻ reverse transcriptase (Gibco BRL, NY, USA). The obtained cDNA served as a template for polymerase chain reaction, consisting of 35 cycles of amplification (95°C for 10 min, 94°C for 50 s, 60°C for 30 s, 72°C for 30 s) with a final elongation of 10 min at 72°C using 0.5 U of Taq DNA polymerase (Promega, Sweden) and 0.5 $\mu\text{mol/L}$ primers (forward: 5'-CGACCTGCTCT GCAAATC-3' and reverse: 5'-CACGCCCACCAGCACGAAGA-3'). PCR using intron-spanning mouse β -actin primers (forward: 5'-CCTGTATGCCTCTGGTTCGTA-3' and reverse: 5'-CCATCTCCTGCTCGAAGTCT-3'), demonstrated that cDNA was present and devoid of genomic DNA contamination. The expected sizes of GHS-R and β -actin fragments were 217 bp and 260 bp, respectively. All primers were selected from conserved regions identified by the alignment of published sequences for GHS-R mRNA in Genbank. PCR products were separated by electrophoresis on 1.4% agarose gels and photos of the separated products were taken.

Statistical analysis

Data are expressed as mean \pm SE. One-way ANOVA was used for statistical analyses of multiple comparisons, and a *P* value of less than 0.05 was considered to be statistically significant.

RESULTS

Contractility in vivo

Compared with the gastric emptying rate of the normal mice (28.10% \pm 1.28%), the gastric emptying rate of the DM mice was significantly reduced (22.90% \pm 1.42%, *P* < 0.05). In DM mice, ghrelin accelerated gastric emptying of the semi-liquid meal at doses of 50, 100 and 200 $\mu\text{g/kg}$; the emptying rate was significantly accelerated from 22.90% \pm 1.42% to 27.80% \pm 0.97%, 34.50% \pm 1.20% and 32.90% \pm 1.10% at doses of 50, 100 and 200 $\mu\text{g/kg}$, respectively (*P* < 0.05, compared to injection of saline) (Figure 1). Similarly, GHRP-6 increased gastric emptying dose-dependently with significant effects at 50, 100 and 200 $\mu\text{g/kg}$ (*P* < 0.05) (Figure 1).

The effect of ghrelin or GHRP-6 on DM mouse gastric emptying was characterized pharmacologically. Ghrelin (100 $\mu\text{g/kg}$) or GHRP-6 (100 $\mu\text{g/kg}$) was unable to reverse the inhibition of gastric emptying due to pretreatment

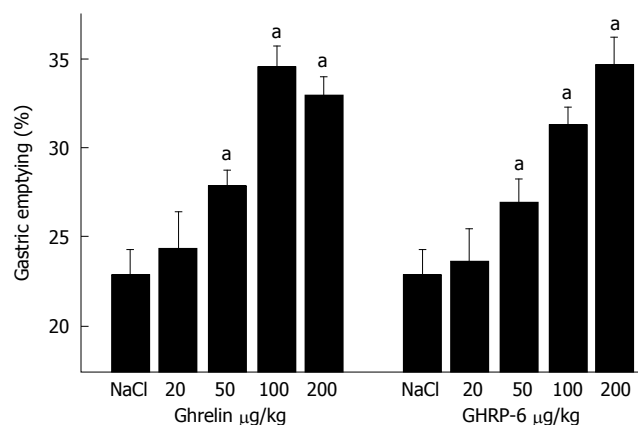


Figure 1 Effect of increasing doses of ghrelin (0–200 $\mu\text{g/kg}$, *ip*) or GHRP-6 on gastric emptying in DM mice. Bars and error bars represent the mean \pm SE of at least six animals. ^a*P* < 0.05 vs normal saline (NaCl).

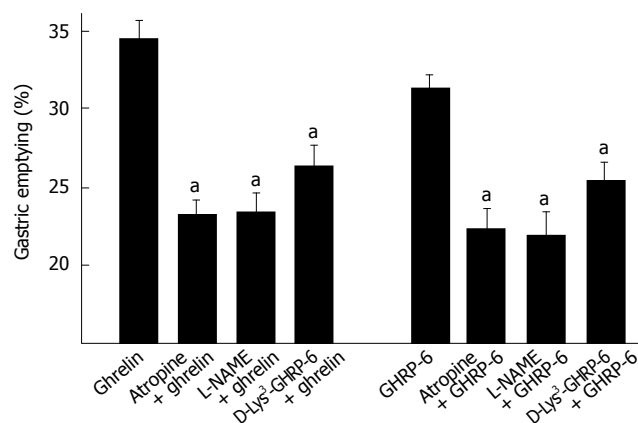


Figure 2 Effects of atropine, L-NAME and D-Lys³-GHRP-6 on the gastroprokinetics of ghrelin or GHRP-6 in the DM mice. Mice were pretreated with atropine (1 mg/kg), L-NAME (50 mg/kg) or D-Lys³-GHRP-6 (5 $\mu\text{mol/kg}$) before administration of ghrelin (100 $\mu\text{g/kg}$) or GHRP-6 (100 $\mu\text{g/kg}$), and the effects were compared with those of treatment with ghrelin or GHRP-6 (100 $\mu\text{g/kg}$) alone. Bar graph and error bars represent the means \pm SE of at least six animals. ^a*P* < 0.05 vs treatment with ghrelin or GHRP-6 (100 $\mu\text{g/kg}$) alone.

with atropine (1 mg/kg) or L-NAME (50 mg/kg) (*P* < 0.05). Pretreatment of DM mice with D-Lys³-GHRP-6 (5 $\mu\text{mol/kg}$) also delayed the accelerated gastric emptying induced by ghrelin or GHRP-6 (*P* < 0.05) (Figure 2).

Contractility in vitro

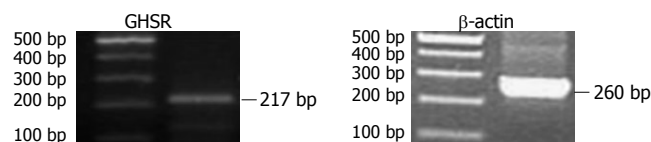
Fundic strips from the DM mice showed spontaneous contractile activity after 1 h of equilibration. Ghrelin (0.01–10 $\mu\text{mol/L}$) or GHRP-6 (0.01–10 $\mu\text{mol/L}$) did not significantly change spontaneous contractile responses in the strips (Table 1). However, in the presence of carbachol (0.1 $\mu\text{mol/L}$), ghrelin increased the carbachol-induced contractile amplitude at 0.1, 1 and 10 $\mu\text{mol/L}$. GHRP-6 also increased the carbachol-induced contractile amplitude at 0.1, 1 and 10 $\mu\text{mol/L}$ (Table 2).

Expression of the ghrelin receptor in mouse fundic strips

The presence of GHS-R mRNA in the mouse fundic smooth muscle strips was verified by RT-PCR with

Table 1 Effects of GHS-R agonists on the spontaneous contractile amplitudes of DM mouse fundic strips

Group	Spontaneous contractile amplitude of DM mouse fundic strips (mg)				
	0	0.01 $\mu\text{mol/L}$	0.1 $\mu\text{mol/L}$	1 $\mu\text{mol/L}$	10 $\mu\text{mol/L}$
Ghrelin	20.4 \pm 1.15	21.3 \pm 0.98	19.8 \pm 1.16	22.1 \pm 1.58	21.5 \pm 1.36
GHRP-6	20.4 \pm 1.15	20.8 \pm 1.12	21.3 \pm 1.74	20.6 \pm 1.27	21.3 \pm 1.35

**Figure 3** Expression of GHS-R mRNA in gastric fundic strips from DM mice. The band at 217 bp corresponds to the amplified GHS-R cDNA product with the expected length. The band at 260 bp corresponds to the amplified β -actin cDNA product with the expected length.**Table 2** Effect of GHS-R agonists on the carbachol (0.1 $\mu\text{mol/L}$)-induced contractile amplitudes of DM mouse fundic strips

Group	Spontaneous contractile amplitude of DM mouse fundic strips (mg)				
	0	0.01 $\mu\text{mol/L}$	0.1 $\mu\text{mol/L}$	1 $\mu\text{mol/L}$	10 $\mu\text{mol/L}$
Ghrelin + carbachol (0.1 $\mu\text{mol/L}$)	60.4 \pm 1.21	61.3 \pm 1.52	65.7 \pm 1.16 ^a	70.0 \pm 1.58 ^a	78.0 \pm 1.56 ^a
GHRP-6 + carbachol (0.1 $\mu\text{mol/L}$)	60.4 \pm 1.21	62.3 \pm 2.14	65.4 \pm 1.24 ^a	72.0 \pm 1.42 ^a	82.0 \pm 1.75 ^a

^a $P < 0.05$, vs carbachol (0.1 $\mu\text{mol/L}$)-induced contraction amplitude of the DM mouse fundic strips.

gene-specific primers. Analysis of the PCR products by electrophoresis revealed a band with the expected length of 217 bp (Figure 3).

DISCUSSION

We have demonstrated, for the first time, that ghrelin and the synthetic peptide GHRP-6 improve gastric emptying in diabetic mice with gastroparesis. This effect may be mediated through potentiation of peripheral cholinergic pathways in the enteric nervous system.

Ghrelin, a recently discovered peptide hormone, is primarily produced by endocrine cells in the oxyntic mucosa of the stomach in rats and humans^[7,12]. Ghrelin has also been found in the small intestine, testis, pituitary gland, ovary, liver, pancreas, kidney, placenta and hypothalamus, in both humans and rodents^[7,11]. Ghrelin is a natural ligand for GHS-R, and its receptor is found all over the body, including in the bowel, pancreas, stomach, heart, lungs and brain^[7,12,20]. In addition to its effect on growth hormone secretion by activating GHS-R in the pituitary gland, ghrelin enhances appetite, increases food intake^[21,22], mediates energy balance, regulates glucose metabolism and insulin release^[23], stimulates gastric acid secretion^[24] and promotes anxiety^[25]. It is well known that many gastrointestinal peptides participate in the regulation of gastrointestinal functions. Ghrelin is one of these candidate gastrointestinal peptides, because it is predominantly present in gastric endocrine cells and is secreted into the bloodstream. In fact, the potential of ghrelin and its synthetic peptide GHRP-6 as a prokinetic agent has been shown previously in *in vitro* and *in vivo* studies. Previous studies on the effect of ghrelin on gastric motility have demonstrated the involvement of vagal and central ghrelin receptors. Thus, the effect of ghrelin on gastric emptying is blocked by atropine and vagotomy in rats and mice^[18,20]. Peripheral ghrelin may stimulate fasted small intestinal motor activity through receptors on vagal afferents, which activate neuropeptide Y-containing

neurons in the brain, as suggested by experiments in rats^[26]. In addition, expression of the ghrelin receptor in the rat nodose ganglion has been confirmed using RT-PCR^[27]. In addition to the known vagal pathways, ghrelin and GHRP-6 accelerate gastric emptying and small intestinal transit by activating cholinergic excitatory pathways in the enteric neuron system^[14,15,17]. Moreover, ghrelin has been shown to increase gastric emptying in patients with gastroparesis, and it has been proposed that ghrelin or its analogues may represent a new class of prokinetic agents for the treatment of gastroparesis^[28,29]. In our study, we investigated the effects of ghrelin and GHRP-6 on gastric motility in diabetic mice with gastroparesis. Our findings indicate the potential of ghrelin as a therapeutic approach for gastrointestinal motility disorders.

In our study, the gastric emptying rate in the DM mice was significantly reduced relative to the normal mice. Ghrelin and GHRP-6 accelerated gastric emptying of the diabetic mice with gastroparesis. In the presence of atropine or L-NAME, which delayed gastric emptying, ghrelin and GHRP-6 (100 $\mu\text{g/kg}$) failed to accelerate gastric emptying. D-Lys³-GHRP-6 also delayed gastric emptying induced by GHS-R agonists. Gastric emptying is a complex process involving excitatory and inhibitory nerves, which may contribute to both acceleration and retardation of the emptying process. L-NAME, which blocks inhibitory nitric nerves, delayed gastric emptying, probably by interfering with gastric accommodation and pyloric relaxation. Therefore, the effect of ghrelin may involve both excitatory and inhibitory pathways, as suggested by the inability of ghrelin to overcome the delay induced by L-NAME and atropine. Ghrelin has been shown to induce release of nitric oxide in the rat stomach, and in our study, a nitric pathway could be involved in the acceleration of gastric emptying because the prokinetic effect *in vivo* was lost in the presence of L-NAME. The GHS-R antagonist D-Lys³-GHRP-6, also blocked the effect of the GHS-R agonists, and this result indicates that the effect of GHS-R agonists on gastric motility

occurs through GHS-R and likely does not involve cross interactions with other receptors. Ghrelin and GHRP-6 increased the carbachol-induced contractile amplitudes in fundic strips taken from DM mice, and this finding also indicates that GHS-R agonists accelerate gastric emptying of semi-liquid through the activation of GHS-R receptors, possibly located on local cholinergic enteric nerves. Moreover, the presence of *GHS-R* mRNA in the strip preparations was confirmed by RT-PCR.

It remains controversial whether ghrelin can exert a protective effect on gastric mucosa, although previous studies have suggested ghrelin might induce gastric mucosal lesion in rats by increasing acid secretion. It is unlikely the improvement in gastric emptying in DM mice induced by ghrelin or GHRP-6 could be explained by a protective effect of ghrelin and GHRP-6 on gastric mucosa. Acid may inhibit gastric emptying, but the effect of ghrelin on acid secretion remains a controversial issue itself^[24,30,31].

In conclusion, ghrelin and its synthetic peptide, GHRP-6, increase gastric emptying in diabetic mice with gastroparesis, perhaps by activating peripheral cholinergic pathways in the enteric nervous system. Although further studies are needed to determine the underlying mechanisms, we propose that ghrelin or its analogues may represent a new class of prokinetic agents for the treatment of diabetic gastroparesis. Therefore, ghrelin and ghrelin agonists have the potential to become useful therapeutic agents for the treatment of diabetic gastroparesis. However, long term animal experiments and clinical trials are needed.

COMMENTS

Background

Delayed gastric emptying is common in patients with chronic diabetes and is always associated with impairments in both quality of life and diabetic control. Ghrelin is a potent prokinetic peptide. Our aim was to test the effect of ghrelin and its synthetic peptide, GHRP-6, on delayed gastric emptying in diabetic mice.

Research frontiers

This study represents the first investigation into the effects of ghrelin and GHRP-6 on diabetic mice with gastroparesis, using both *in vivo* and *in vitro* approaches.

Related publications

Ghrelin has been under intensive study for its effects on gastrointestinal motor activity and its roles in motility regulation. In addition to influencing food intake and energy balance, ghrelin also possesses prokinetic characteristics mediated by the activation of cholinergic pathways.

Innovations and breakthroughs

Ghrelin has been shown to accelerate gastric emptying in postoperative and septic ileus animal models. However, it has not been studied in a diabetic animal model.

Applications

According to our effective therapy in animal experiments, ghrelin and ghrelin agonists may have the potential to become useful therapeutic agents for the treatment of diabetic gastroparesis.

Terminology

Gastroparesis: A condition of delayed stomach emptying, often seen as a complication of diabetes mellitus.

Peer review

This paper investigated for the first time *in vivo* and *in vitro* that ghrelin and its

synthetic peptide, GHRP-6, improves gastric emptying in diabetic mice with gastroparesis, and this effect may be mediated through peripheral cholinergic pathways in the enteric nervous system. These results are potentially significant for the clinical treatment of diabetic gastroparesis.

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Requirements for transfusion and postoperative outcomes in orthotopic liver transplantation: A meta-analysis on aprotinin

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Abstract

AIM: To study the effect of aprotinin used in orthotopic liver transplantation (OLT) on the intraoperative requirement for blood products and on the incidence of laparotomy for bleeding, thrombotic events and mortality.

METHODS: A systematic review of the literature in the electronic database Medline and the Clinic Trials Registry Database was performed. Literature that did not fit our study were excluded. Patients in the reviewed studies were divided into two groups; one group used aprotinin (aprotinin group) while the other did not (control group). The data in the literature that fit our requirements were recorded. Weighted mean differences (WMD) in the requirements for blood products between the aprotinin group and the control group were tested using a fixed effect model. A Z test was performed to examine their reliability; the Fleiss method of fixed effect model was used to analyze data on postoperative events, and odds ratios (ORs) were tested and merged.

RESULTS: Seven citations were examined in our study. Among them, a requirement for blood products was reported in 4 studies including 321 patients, while postoperative events were reported in 5 studies including 477 patients. The requirement for red blood cells and fresh frozen plasma in the aprotinin group was statistically lower than that in the control group (WMD = -1.80 units, 95% CI, -3.38 to -0.22; WMD = -3.99 units, 95% CI, -6.47 to -1.50, respectively). However, no significant difference was indicated in the incidence of laparotomy for bleeding, thrombotic events and mortality between the two groups. Analysis on blood loss, anaphylactic reactions and renal function was not performed in this study due to a lack of sufficient information.

CONCLUSION: Aprotinin can reduce the intraoperative requirement for blood products in OLT, and has no significant effect on the incidence of laparotomy for bleeding, thrombotic events and mortality.

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Key words: Aprotinin; Liver transplantation; Blood transfusion; Meta-analysis

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INTRODUCTION

Orthotopic liver transplantation (OLT) has become the first choice approach for the treatment of patients with end-stage liver diseases^[1]. However, despite great improvements in graft preservation, surgical skills, anesthetic techniques and perioperative management^[2,3], OLT is still associated with severe bleeding and considerable transfusion requirements, which in turn greatly contribute to the peri-operative morbidity and mortality^[4]. Severe bleeding in OLT occurs for several reasons, among which hemostatic abnormalities remain a major cause^[5,6].

Aprotinin, a serine protease inhibitor, is more and more commonly being used in surgeries, such as cardiac surgeries and liver transplantations, to reduce bleeding and the need for transfusions. A meta-analysis of 12 trials ($n = 626$) of children undergoing cardiac surgery demonstrated aprotinin reduced the proportion of children receiving blood transfusions during cardiac surgery with cardiopulmonary bypass, but had no significant effect on the volume of blood transfused or on the amount of chest tube drainage^[7]. Similarly, a meta analysis of 13 trials ($n = 506$) of patients undergoing major orthopedic surgery demonstrated the pooled blood loss and the amounts of red blood cell (RBC) units (U) transfused intraoperatively and peri-operatively were significantly lower among aprotinin-treated patients than control patients. Moreover, aprotinin was not associated with an increased incidence

of deep vein thrombosis^[8]. However, there are still some conflicting results on whether aprotinin can reduce blood loss or the requirement for transfusion in OLT^[9,10], and whether it can be beneficial to postoperative outcomes^[11,12]. The objective of this systemic review was to study the effect of aprotinin used in OLT on the intraoperative requirement of blood products, and on the incidence of laparotomy for bleeding, thrombotic events and mortality.

MATERIALS AND METHODS

Data source

We searched the electronic database of Medline and the Clinic Trials Registry Database using aprotinin and liver transplantation as keywords. References cited by other retrospective articles and related articles or summaries from foreign journals were searched manually as well. After initial screening, we examined the titles and abstracts of potentially eligible trials, and selected those which met the following predefined inclusion criteria: published clinical controlled trials on the use of aprotinin in liver transplantation, English language, adult study population, with data on (1) the transfusion requirement for blood products, (2) perioperative mortality and morbidity, (3) incidence of postoperative thrombotic events and (4) incidence of laparotomy for bleeding. Citations that did not fit our study or contained insufficient information were excluded.

Statistical analysis

We recorded the data that fit our requirements, examined their heterogeneity, and calculated the weighted mean difference (WMD) or odds ratio (OR) between the two groups. All calculations were performed using the software Review Manager 4.2 (The Nordic Cochrane Centre, The Cochrane Collaboration 2003, Copenhagen, Denmark).

We used the difference of means (y_i) as the effect scale of the data on requirements of blood products and examined their heterogeneity ($Q < \chi^2_{(0.05, k-1)}$, $P > 0.05$), if $P > 0.05$, fixed effect model was used to calculate WMD and 95% confidence interval (95% CI); otherwise, a random effect model was used. If the 95% CI included 0, then there was no significant difference between the two groups. However, if the 95% CI was greater than 0, then the control group was supported; otherwise, the aprotinin group was supported.

We calculated the ORs of the incidence of mortality, laparotomy for bleeding and thrombotic events, and tested their heterogeneity ($Q < \chi^2_{(0.05, k-1)}$, $P > 0.05$). If $P > 0.05$, the homogeneity was considered good, and a fixed effect model was used to calculate the total OR and 95% CI; otherwise a random effect model was chosen. If 1 was included in the 95% CI, then there was no statistically significant difference between the groups. If the 95% CI was more than 1, the control group was supported; otherwise, the aprotinin group was supported.

RESULTS

Recording of data

We identified 87 citations in a primary literature search.

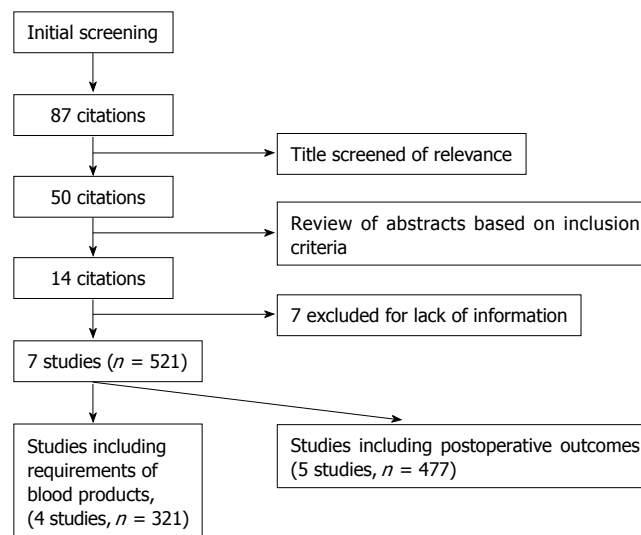


Figure 1 Results of article search and selection.

Titles were screened for relevance, eliminating 37 citations, and then abstracts and contents were read carefully, leading to the exclusion of a further 36 citations; 7 more were excluded because of a lack of information. Finally 7 citations^[9,10,13-17], including 521 patients, were included in our study (Figure 1). Of these 7 studies, one used tranexamic acid in the control group^[16]; two studies contained two aprotinin groups, a high dose group and a routine dose group^[10,15]; one study contained two control groups^[17]; and two studies used the same sample^[10,15], the size of which was calculated only once.

Effect of aprotinin on RBC requirement

Four citations, including 321 procedures, contained results on the requirement for blood products including RBCs and fresh frozen plasma (FFP)^[10,14,16,17].

One of these studies contained two control groups^[17] (C1 and C2); Neither aprotinin nor any other antifibrinolytic agent was used in either group, so we just took C2 as the control group. Heterogeneity was tested: $Q = 8.87$, $\gamma = 3$, $\chi^2_{(0.05, 3)} = 7.81$, $P < 0.05$. As the homogeneity was low, a random effect model was used: WMD = -1.23 units, 95% CI, -3.17 to 0.71; no statistical significance was indicated. Considering one study used tranexamic acid in control group^[17], it perhaps influenced the veracity, so we excluded that study and tested again, $Q = 3.85$, $\gamma = 2$, $\chi^2_{(0.05, 2)} = 5.99$, $P > 0.05$, calculated with fixed effect model. It was indicated the intraoperative requirement for RBCs was significantly lower in the aprotinin group than the control group (WMD = -1.80 units, 95% CI -3.38 to -0.22; Table 1A and B).

Effect of aprotinin on FFP requirement

The heterogeneity of the 4 citations was low ($Q = 13.77$, $\gamma = 3$, $\chi^2_{(0.05, 3)} = 7.81$, $P < 0.05$), so a random effect model was used. No significant difference was indicated (WMD = -3.13 units, 95% CI -6.79 to 0.53). If the study using individuals treated with tranexamic acid as a control group was excluded, the heterogeneity was better ($Q = 5.25$, $\chi^2_{(0.05, 2)} = 5.99$, $P > 0.05$), and a fixed effect model was chosen. It was indicated the intraoperative requirement for

Table 1A Volumes of RBCs transfused intraoperatively in the 4 studies

Study	Aprotinin group			Control group			y_i (95% CI)
	Mean	SD	<i>n</i>	Mean	SD	<i>n</i>	
	(units)	(units)		(units)	(units)		
1 Garcia HL	13.0	8.0	39	14.4	9.7	41	-1.4 (-5.29, 2.49)
2 Marcel RJ	2.1	2.0	21	3.0	4.4	23	-0.9 (-2.89, 1.09)
3 Dalmau A ¹	2.44	3.03	63	2.14	2.32	64	0.3 (-0.64, 1.24)
4 Llamas P	8.1	5.2	20	13	7.4	30	-4.9 (-8.39, -1.41)

¹Control group used tranexamic acid.**Table 2A** Volumes of FFP transfused intraoperatively in the 4 studies

Study	Aprotinin group			Control group			y_i (95% CI)
	Mean	SD	<i>n</i>	Mean	SD	<i>n</i>	
	(units)	(units)		(units)	(units)		
1 Garcia HL	26.0	16.0	39	28.0	15.0	41	-2.00 (-8.80, 4.80)
2 Marcel RJ	3.6	3.5	21	6.6	6.1	23	-3.00 (-5.91, -0.09)
3 Dalmau A ¹	1.09	2.20	63	1.20	2.21	64	-0.11 (-0.88, 0.66)
4 Llamas P	16.7	10.4	20	28	14	30	-11.3 (-18.07, -4.53)

¹Control group used tranexamic acid.**Table 1B** Weighted mean differences (WMDs) in the volumes of RBCs transfused intraoperatively

	Heterogeneity		WMD (units)	Z test		95% CI
	Q	P		Z	P	
A	8.87	0.03	-1.23	1.24	0.21	-3.17 to 0.71
B	3.85	0.15	-1.80	2.23	0.03	-3.38 to -0.22

A: Total results; B: Results with study No. 3 excluded.

Table 2B Weighted mean differences (WMDs) in the volumes of FFP transfused intraoperatively

	Heterogeneity		WMD (units)	Z test		95% CI
	Q	P		Z	P	
A	13.77	0.003	-3.13	1.68	0.09	-6.79 to 0.53
B	5.25	0.07	-3.99	3.14	0.002	-6.47 to -1.50

A: Total results; B: Results with study No. 3 excluded.

Table 3A Postoperative outcomes of the 5 studies

Study	Aprotinin group				Control group				OR _{i1} , 95% CI	OR _{i2} , 95% CI	OR _{i3} , 95% CI
	<i>n</i>	<i>n1</i>	<i>n2</i>	<i>n3</i>	<i>n</i>	<i>n1</i>	<i>n2</i>	<i>n3</i>			
1 James Y	33	0	1	1	30	1	4	2	0.29 (0.01, 7.48)	0.20 (0.02, 1.93)	0.44 (0.04, 5.09)
2 Garcia HL	39	1	2		41	1	3		1.05 (0.06, 17.43)	0.68 (0.11, 4.34)	
3 Porte RJ	89	5	7	2	48	5	2	3	0.51 (0.14, 1.87)	1.96 (0.39, 9.85)	0.34 (0.06, 2.14)
4 Dalmau A ¹	63	1	2	2	64	4	2	4	0.24 (0.03, 2.23)	1.02 (0.14, 7.45)	0.49 (0.09, 2.79)
5 Llamas P	20	4	0		50	6	7		1.83 (0.46, 7.35)	0.14 (0.01, 2.60)	

n1: Number of deaths; *n2*: Number of laparotomy for bleeding; *n3*: Number of thromboembolic events; ¹Control group used tranexamic acid.**Table 3B** Odds ratios of the postoperative outcomes

		Heterogeneity of OR _i		OR	χ^2 test		95% CI
		Q	P		χ^2	P	
Death	A	3.32	0.51	0.69	0.48	> 0.05	0.32-1.52
	B	2.21	0.53	0.85	0.06	> 0.05	0.36-2.00
Laparotomy for bleeding	A	4.09	0.39	0.65	0.44	> 0.05	0.29-1.43
	B	3.94	0.27	0.59	0.57	> 0.05	0.25-1.41
Thrombotic events	A	0.08	0.96	0.42	2.33	> 0.05	0.14-1.30
	B	0.02	0.88	0.38	1.75	> 0.05	0.09-1.64

A: Total results; B: Results with study No. 4 excluded.

FFP was significantly lower in the aprotinin group than in the control group (WMD = -3.99 units, 95% CI -6.47 to -1.50; Table 2A and B).

Effect of aprotinin on postoperative outcomes

As can be seen from Table 3A and B, no significant difference was indicated in the incidence of laparotomy for bleeding, thromboembolic events and mortality between the two groups.

DISCUSSION

Unlike traditional reviews, a meta analysis is a set of statistical procedures designed to accumulate experimental and correlational results across independent studies which address related sets of research questions. The aim of the meta-analysis is to determine a predefined inclusion criteria based on the systematic retrieval of literature on a given topic, and estimate the initial literatures carefully to ensure minimal bias in terms of the objectivity, validity and dependability of the results. The efficiency of the results depends on the choice of statistical method, as well as the rigidity of each study. In this meta analysis, we performed a wide search of the literature, identified as many studies as we could, and tested their heterogeneity ($Q < \chi^2_{(0.05, k-1)}, P > 0.05$). If homogeneity was good ($P > 0.05$), we calculated data with a fixed effect model; otherwise we used a random effect model. Thus, we consider the statistical methods we used were correct and rigorous.

Several factors contribute to excessive bleeding during OLT, including pre-existing coagulopathy in patients^[18-20], the procedure of liver transplantation itself, and the experience of the surgeon. However, hemostatic

abnormalities due to hyperfibrinolysis remain a major cause. Hyperfibrinolysis always occurs late in the anhepatic phase and immediately after the reperfusion of the graft^[21]. This enhanced fibrinolytic activation is due to an excess of tissue-type plasminogen activator (t-PA) on account of the lack of hepatic clearance and its increased release from the ischemically damaged endothelium, associated with the consumption of α_2 -antiplasmin (α_2 -AP) and plasminogen activator inhibitor type 1 (PAI 1)^[22-24]. Using a suitable method to protect blood and control the coagulopathy of patients can not only reduce the need for transfusions, reduce the transmission of diseases and immunological reactions due to the transfusion of banked blood, but also have great benefit to peri-operative hemodynamic stability. Several methods can be used to protect blood during liver transplantation, including transfusion of autologous blood, appropriate body temperature and perioperative use of blood protective drugs. Aprotinin, a basic polypeptide and non-specific proteinase inhibitor, can inhibit several proteases with serine active groups. Recently, aprotinin became no longer restricted to the treatment of patients with acute pancreatitis; it is being used more and more in cardiac surgeries, orthopedic surgeries and liver transplantations^[25,26], and is considered the ideal blood protecting drug.

Aprotinin inhibits kallikrein, reduces the release of callidin and results in a decrease in the level of t-PA. In a larger, randomized, double-blind, placebo-controlled study, Molenaar *et al*^[27] compared coagulation [fibrinogen level, activated partial thromboplastin time (aPTT), prothrombin time and platelet count] and fibrinolytic variables (tPA antigen and activity, plasminogen activator inhibitor activity and D-dimer), as well as thromboelastography results [reaction time (R), clot formation time, and maximum amplitude] between an aprotinin group and a placebo group. They found fibrinolytic activity (plasma D-dimer and tPA antigen levels) was significantly lower in aprotinin-treated patients compared with the placebo group, but coagulation times (aPTT and R) were significantly more prolonged. It was indicated aprotinin has an anticoagulant rather than a procoagulant effect. Its blood-sparing (prohemostatic) effect appears to be the overall result of a strong antifibrinolytic and a weaker anticoagulant effect. In our study, it was found that, when normal saline or a placebo was used in the control group, the requirement for red blood cells was significantly lower in the aprotinin group than in the control group (WMD = -1.80 units, 95% CI, -3.38 to -0.22 units; moreover, the requirement for fresh frozen plasma was also significantly lower in the aprotinin group than in the control group (WMD = -3.99 units, 95% CI, -6.47 to -1.50). However, no significant difference was indicated in the incidence of laparotomy for bleeding, thromboembolic events, and mortality between the two groups. It was demonstrated aprotinin can reduce the intraoperative requirement for blood products in OLT. The effect of aprotinin on blood loss during OLT was not reviewed in this study because of a lack of information; more clinic trials are needed for advanced investigation.

As an extraneous protein, aprotinin causes allergies, which can induce typical allergic reactions in patients^[28,29], especially those who use it again. Aprotinin is mainly

metabolized by the kidney, and it has nephrotoxicity if used at high doses. The serum creatinine levels in patients using aprotinin during an operation increased at 3-5 d postoperatively, indicating an influence on renal function^[14]. However, it was also reported the number of patients whose serum creatinine levels increased by more than 5 mg/L was lower in the aprotinin group than in the control group. No significant difference was found in peri-operative creatinine clearance rates^[30].

There are three causes of thromboembolic events: Injury of blood vessels, changes in the blood stream, and coagulation state. It was reported aprotinin can lead to intravascular thrombosis and thromboembolism during liver transplantation^[31]. In our study, the OR of thrombosis was 0.42, the 95% CI was 0.14 to 1.30, and there was no significant deviation; While the study using TA as control group was excluded, the OR was 0.38, the 95% CI was 0.09 to 1.64, and there was also no statistically significant deviation. It appears aprotinin has no significant influence upon the incidence of thrombosis in patients undergoing liver transplantation, possibly due to its strong antifibrinolytic and a weaker anticoagulant effect.

As a statistical method for investigation, meta analysis has been used widely, but this method can not eliminate confounding factors and biases in each study, so the result could, unavoidably, include a certain bias. In our study, the dosages of aprotinin were different, and the drugs used in the control groups were not the same, all of which could contribute to bias. In addition, there still exists the "publish bias"; that is, articles that are published often have a tendency to have positive results, which could be decreased by collecting data that is as all-encompassing as possible.

Thus, aprotinin can reduce the intraoperative requirement for blood products in OLT and has no significant effect on the incidence of laparotomy for bleeding, thromboembolic events and mortality. Of course, further clinical randomized controlled trials (RCTs) are needed to confirm this.

COMMENTS

Background

Orthotopic liver transplantation (OLT) is associated with severe bleeding and considerable transfusion requirements, while severe bleeding in OLT occurs for several reasons, among which hemostatic abnormalities remain a major cause.

Research frontiers

We performed a meta analysis to study the effect of aprotinin used in OLT on the intraoperative requirement for blood products and the postoperative outcomes.

Innovations and breakthroughs

This study clearly shows aprotinin can reduce the intraoperative requirement for blood products and has no significant effect on the incidence of laparotomy for bleeding, thromboembolic events and mortality.

Applications

Although additional clinical randomized controlled trials (RCTs) are required to clarify the role of aprotinin in OLT, this study strongly confirms the blood transfusion reducing effect of aprotinin, which has no significant effect on the incidence of laparotomy for bleeding, thromboembolic events and mortality.

Peer review

The authors investigated the effect of aprotinin used in OLT on the intraoperative

requirement of blood products and on the incidence of laparotomy for bleeding, thrombotic events and mortality, using a systematic review of the literature. They concluded aprotinin can reduce the intraoperative requirement of blood product in OLT, and has no significant effect on the incidence of laparotomy for bleeding, thrombotic events and mortality.

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RAPID COMMUNICATION

Percutaneous cryosurgery for the treatment of hepatic colorectal metastases

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Abstract

AIM: To determine the safety and efficacy of efficacy of percutaneous cryosurgery for treatment of patients with hepatic colorectal metastases.

METHODS: Three hundred and twenty-six patients with non-resectable hepatic colorectal metastases underwent percutaneous cryosurgery under the guidance of ultrasound or CT. Follow-up was 1 mo after cryosurgery and then every 4 mo thereafter by assessment of tumor markers, liver ultrasonography, and abdominal CT. For lesions suspicious of recurrence, a liver biopsy was performed and subsequent repeat cryosurgery was given if histology was positive for cancer.

RESULTS: All patients underwent a total of 526 procedures of cryosurgery. There were 151 patients who underwent repeat procedures of cryosurgery for recurrent tumors in the liver and extrahepatic places. At 3 mo after cryosurgery, carcinoembryonic antigen (CEA) levels in 197 (77.5%) patients who had elevated markers before cryosurgery decreased to normal range. Among 280 patients who received CT following-up, cryotreated lesions showed complete response (CR) in 41 patients (14.6%), partial response (PR) in 115 patients (41.1%), stable disease (SD) in 68 patients (24.3%) and progressive disease (PD) in 56 patients (20%). The recurrence rate was 47.2% during a median follow-up of 32 mo (range, 7-61). Sixty one percent of the recurrences were seen in liver only and 13.9% in liver and extrahepatic areas. The recurrence rate at cryotreated site was only 6.4% for all cases. During a median follow-up of 36 mo (7-62 mo), the median survival of all patient was 29 mo (range 3-62 mo). Overall survival was 78%, 62%, 41%, 34% and 23% at 1, 2, 3, 4 and 5 years, respectively, after the treatment. Patients with tumor size less than 3 cm, tumor in right lobe of liver, lower CEA levels (< 100 ng/dL) and post-cryosurgery TACE had higher survival rate. There was

no significant difference in terms of survival based on the number of tumors, pre-cryosurgery chemotherapy and the timing of the development of metastases (synchronous vs metachronous). Patients who underwent 2-3 procedures of cryosurgery had increased survival compared to patients who received cryosurgery once only. There was no intra-cryosurgery mortality. Main adverse effects, such as hepatic bleeding, cryoshock, biliary fistula, liver failure, renal insufficiency and liver abscess were only observed in 0.3%-1.5% of patients.

CONCLUSION: Percutaneous cryosurgery was a safe modality for hepatic colorectal metastases. Rather than an alternative to resection, this technique should be regarded as a complement to hepatectomy and as an additional means of achieving tumor eradication when total excision is not possible.

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Key words: Hepatic colorectal metastases; Hepatic cryosurgery; Percutaneous cryosurgery; Colorectal cancer

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INTRODUCTION

Hepatic metastasis is the main cause of death in patients with colorectal carcinoma. Hepatic resection is the treatment of choice for liver cancer if metastases are confined to the liver, and may achieve a 5 years survival of 25%-39%^[1-4]. Resectability is usually determined by the absence of extrahepatic metastases, a maximum of four lesions in the liver and the ability to obtain cancer-free resection margins^[5]. Therefore, only 10%-20% of patients with hepatic colorectal metastases are suitable

for resection^[6]. For non-resectable liver metastases, chemotherapy or chemoembolization are often used, but the outcome is poor. Median survival is around 12 mo for non-resectable hepatic colorectal metastases^[7].

Cryosurgery has recently been applied to non-resectable liver tumors, and has shown encouraging results^[8-11]. Between March 2001 and February 2007, 1090 patients with malignant liver tumors were treated by cryosurgery in our hospital. There were 680 patients with hepatocellular carcinoma (HCC), 326 with liver metastases originating from colorectal carcinoma, and 84 with liver metastases from cancer of non-colorectal origin. This study describes the results of percutaneous cryosurgery for the treatment of non-resectable hepatic colorectal metastases, with the purpose of determining the efficacy and safety of this modality.

MATERIALS AND METHODS

Patients

Three hundred and twenty six patients with hepatic colorectal metastases were enrolled in this study. There were 243 men and 83 women with a mean age of 54.8 years (range, 32-84). Patient and tumor characteristics are listed in Table 1.

Diagnosis of hepatic colorectal metastases was made by intraoperative findings during colectomy, and in postoperative follow-up, by the combination of increased levels of tumor markers [carcinoembryonic antigen (CEA)] and imaging of lesions by ultrasonography, CT or MRI of the liver. There were 234 patients whose diagnosis was proven by liver biopsy. Liver metastases were synchronous in 65 cases (19.9%) and metachronous in 261 cases (80.1%). The tumors of all cases received a thorough investigation with regard to the presence of multiple nodules or to the presence of a large and/or ill-located tumor, comorbidity, and were considered as non-resectable. Patients with extrahepatic metastases or liver failure were excluded from this study.

All patients were given cryosurgery guidelines, and the study received ethical approval.

Cryosurgery technique

Cryosurgery was performed with Cryocare Operative System (Endocare, CA, USA) which used the Joule-Thomson effect to cool the end of a cryoprobe in closed systems. In accordance with the gas coefficient and the dimension of the nozzle, different gaseous elements generate different thermal exchange events at the area close to the nozzle. Argon gas is used for cooling (-187°C), and helium is used for heating (67°C). The probe was inserted percutaneously under ultrasound or CT guidance, and two freezing-thawing cycles were performed. We used mainly 2- or 5-mm probes and rarely a 10-mm probe, according to the size of the tumor. Two or more probes were used simultaneously for large lesions. Individual tumors may be frozen sequentially on a tumor-by-tumor basis or simultaneously. The time of freezing was dependent on the achievement of an "ice-ball", visible as a hypoechogenic area on ultrasonography, > 1 cm the diameter of the lesion. Thawing was achieved by input

Table 1 Characteristics of patients with hepatic colorectal metastases

Total cases	326
Median age (range)	54 (32-84)
Male/Female (cases)	243/83
No. of tumors (cases)	
1	125 (38.3%)
2	105 (32.2%)
3	65 (19.9%)
More	31 (9.5%)
Tumor size (cases)	
< 3 cm	95 (29.1%)
3-5 cm	124 (38.0%)
> 5 cm	107 (32.8%)
Development of metastases	
Synchronous (cases)	65 (19.9%)
Metachronous (cases)	261 (80.1%)
Colectomy to detection of metastases (mo)	12 (0-42)
Metastases detected to cryosurgery (mo)	4 (1-14)
Colectomy to cryosurgery (mo)	16 (3-52)
Precryosurgery chemotherapy(cases)	216 (66.3%)
Precryosurgery CEA (mg/dL)	11.2 (0.3-1422)

of helium during a period equivalent to the freezing time before the second freezing process was begun. Hemostasis of the insertion hole of the cryoprobe was obtained by Spongel application to the tract of the cryoprobe and by suture of the insertion site.

Transarterial chemoembolization (TACE)

After cryosurgery, 280 patients underwent one or two sessions of TACE within 1-2 mo. The reasons for using TACE were larger tumors prior to cryosurgery, multiple tumors, or higher CEA level after cryosurgery. The chemotherapeutic agents for intra-arterial infusion were a mixture of Lipiodol and doxorubicin, cisplatin, 5-fluorouracil, and mitomycin C. Occasionally gelfoam was used as an embolization material.

Follow-up

Postoperative follow-up was at the first month and then every 4 mo after cryosurgery, by assessment of liver function tests, tumor markers, liver ultrasonography, and abdominal CT. Some of patients received follow-up with positron emission tomography (PET). Efficacy of cryosurgery for tumors was evaluated according to the evolution of tumor size and tumor markers. Changes in tumor mass were measured according to the Response Evaluation Criteria in Solid Tumors (RECIST) protocol^[12], which is based on objective measurement of lesion size before and after treatment. Complete response (CR) means cryotreated lesion disappearance (scar) or < 25% of original size. Partial response (PR) means a > 30% decrease in the sum of the largest diameter of all targeted lesions. Stable disease (SD) means < 30% decrease in the sum of the largest diameter of all targeted lesions. Progressive disease (PD) means an increase of > 20% in the sum of the largest diameter of all targeted lesions.

All radiologic studies were reviewed by the same radiologist with expertise in hepatic imaging. For lesions suspicious of recurrence, an ultrasound-guided liver biopsy was performed for histological study. Subsequent repeat

Table 2 Sites of repeat cryosurgery

Cryosurgery sites	No. of patients	Procedures of repeat cryosurgery
Liver only	105	142
Liver and lungs	40	52
Liver and pancreas	6	6
Total	151	200

Table 3 Recurrence pattern at death or latest follow-up

	Cases <i>n</i> (%)	% of all cases
Total recurrence	136 (100)	41.7
Liver only	83 (61.0)	25.5
Cryosite only	7 (5.1)	2.1
Liver other than cryosite only	62 (45.5)	19.0
Cryosite and remaining areas	14 (10.2)	4.3
Extrahepatic metastases only	34 (25.0)	10.4
Lungs	10 (7.4)	3.1
Brain	2 (1.4)	0.6
Bone	4 (2.9)	1.2
Lymph nodes	11 (8.1)	3.4
Peritoneum	3 (2.2)	0.9
Multiple areas	4 (2.9)	1.2
Liver and some extrahepatic areas	19 (13.9)	5.8
Liver and lungs	8 (5.8)	2.8
Liver and pancreas	2 (1.4)	0.7
Liver and lymph nodes	5 (3.7)	1.7
Liver and bone	1 (0.7)	0.3
Liver and others	3 (2.2)	1.0

cryosurgery was performed if histology was positive for cancer. A persistent nodule on radiological imaging, without tumor activity shown on PET, or with reduced or normal tumor markers (CEA), or no changes of the nodule size within at least 6 mo since cryosurgery, was considered as a remnant. Tumor recurrence was estimated either by histological examination of the liver, or by combination of size increase of the cryotreated lesion on ultrasound, CT or PET imaging and increased tumor markers.

Statistical analysis

Survival was determined according to the Kaplan-Meier method. Comparison of survival rates was obtained with the log-rank test. $P < 0.05$ was considered statistically significant.

RESULTS

All patients underwent a total of 526 procedures of cryosurgery. There were 175 patients who received a single procedure of cryosurgery, and 151 patients who underwent repeat procedures of cryosurgery for recurrent tumors in the liver and extrahepatic places (Table 2).

Changes in tumor marker

Increased CEA level was observed in 254 patients (77.9%) at the time of the initial diagnosis. Among these patients, CEA level decreased to within the normal range in 197 (77.5%) patients, and increased in 41 patients (16.1%), with no significant change in 16 cases (6.3%), at 3 mo after cryosurgery.

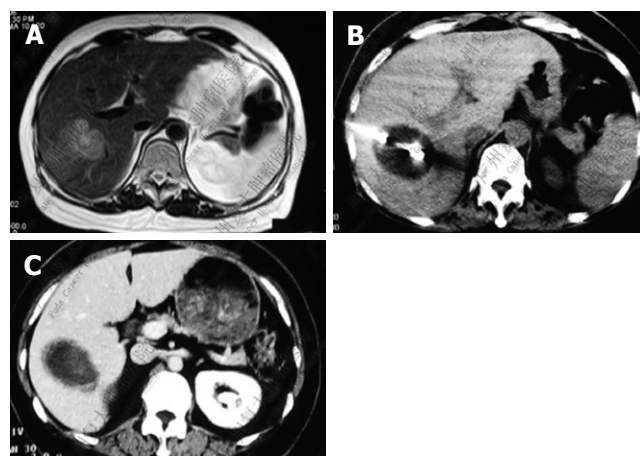


Figure 1 Liver imaging in patients with hepatic colorectal metastases. Complete ablation of histology-proven tumor achieved after percutaneous cryosurgery. A: MRI before cryosurgery; B: During percutaneous cryosurgery under CT guidance; C: Twelve mo after cryosurgery.

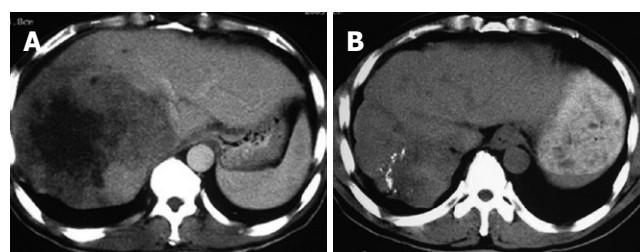


Figure 2 CT of a patient with hepatic colorectal metastases treated by percutaneous cryosurgery. The massive lesion showed a CR to cryosurgery and TACE. A: Before cryosurgery; B: Eight months after cryosurgery and TACE.

Evolution of tumor size

After cryosurgery, an early increase in the size of lesions in relation to the freezing margin > 1 cm beyond the limit of the tumor was a constant feature. Cryotreated lesions appeared as hypoechogenic or hypodense areas. Among 280 patients who received CT follow-up, CR was observed in 41 patients (14.6%), PR in 115 (41.1%), SD in 68 (24.3%), and PD in 56 patients (20%). Two patients with CR proven by histology and CT are presented in Figures 1 and 2.

Tumor recurrence

The recurrence rate was 47.2% during a median follow-up of 32 mo (range, 7-61). Recurrence patterns are presented in Table 3. Sixty-one percent of recurrence was in the liver only and 13.9% in the liver and extrahepatic areas. Extrahepatic recurrence was mainly seen in the lungs and lymph nodes. The recurrence at cryotreated site, including at cryotreated site only as well as cryotreated site and the remaining area of liver, accounted 15.3% of cases who had recurrence and 6.4% of all cases.

Overall survival

During a median follow-up of 36 mo (7-62 mo), the median survival of all patient was 29 mo (range 3-62 mo). One hundred and ninety six patients (60.1%) died during follow-up, and 130 patients (39.9%) are still alive, with a median survival of 26 and 36 mo, respectively. Overall

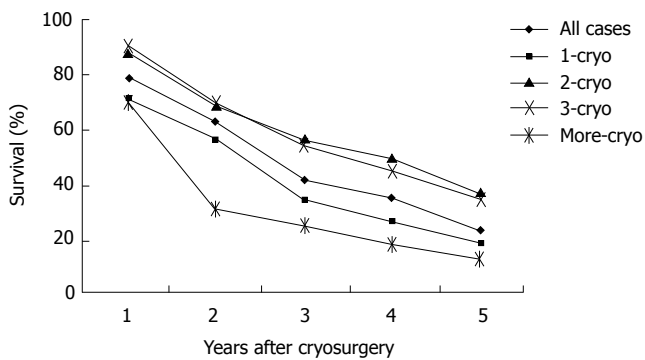


Figure 3 Patient survival after cryosurgery.

Table 4 Survival based on patient characteristics and tumor features

	Median survival (mo)	Survival rate (%)		
		1 yr	3 yr	5 yr
All patients	29	78	41	23
Tumor size				
≤ 3 cm ^b	39	92	64	34
> 3 cm	21	70	41	21
Tumor number				
< 3	38	81	40	20
> 3	40	78	41	22
Tumor location				
Right lobe ^b	33	87	57	39
Left lobe	25	72	39	21
Bilobar	17	65	32	14
Detection of metastases				
Synchronous	30	76	43	24
Metachronous	29	81	41	21
Metastases detected to cryosurgery				
< 3 mo	31	74	41	22
> 3 mo	29	79	42	23
Pre-cryosurgery chemotherapy				
Yes	30	75	40	24
No	29	77	41	21
Pre-cryosurgery CEA				
< 100 ng/dL ^b	44	90	57	41
> 100 ng/dL	20	73	39	17
Cryosurgery procedure				
Once	21	70	34	19
Twice ^b	38	87	55	36
Thrice ^b	39	90	54	34
More	12	69	25	13
Post-cryosurgery TACE				
Yes ^b	38	84	57	47
No	18	76	25	15

^bP < 0.01 Survival of patients with tumor size ≤ 3 cm *vs* > 3 cm, tumor location right lobe *vs* left lobe or bilobar, pre-cryosurgery CEA < 100 ng/dL *vs* > 100 ng/dL, twice or thrice procedures *vs* once or more procedures, and TACE *vs* no TACE after cryosurgery.

survival was 78%, 62%, 41%, 34% and 23% at 1, 2, 3, 4 and 5 years, respectively (Figure 3). Patients with tumor ≤ 3 cm, tumor in right liver lobe, CEA < 100 ng/dL, and post-cryosurgery TACE had a higher survival rate. There was no significant difference in terms of survival based on tumor number, pre-cryosurgery chemotherapy, and timing of the development of metastases (synchronous *vs* metachronous) (Table 4). Survival was related to the number of cryosurgery procedures performed on the patients. Patients who

Table 5 Adverse effects within 30 d after cryosurgery

Adverse effects	No. of patients	%
Minor		
Pain	103	31.6
Fever (> 38°C)	108	33.1
Increased liver enzymes	124	38
Thrombocytopenia	58	17.8
Pleural effusion	20	6.1
Major		
Hepatic bleeding	5	1.5
Cryoshock	1	0.3
biliary fistulae	3	0.9
Liver failure	1	0.3
Renal insufficiency	5	1.5
Liver abscess	3	0.9
Acute myocardial infarction and severe arrhythmias	2	0.6

underwent two or three procedures had an increased survival, compared to those who received cryosurgery only once. However, patients who received cryosurgery on more than three occasions had lower survival (Table 4, Figure 3).

Mortality and morbidity

The minor and major adverse effects of cryosurgery are shown in Table 5. A temporary pain in the abdominal right-upper quadrant and fever (about 38°C) were observed in about half the patients. An elevation of serum transaminase levels occurred in 124 patients and normalization was observed within 14 d. Differing degrees of thrombocytopenia were seen in 58 patients, only four of whom received infusion of fresh frozen plasma or platelet concentrate, none had poor consequence. Twenty patients, of whom, 18 had sub-diaphragmatic liver tumor, had right pleural effusion, probably due to the irritative process beneath the diaphragm.

There was no cryosurgical mortality. A total of 19 patients developed major adverse effects. Hemorrhage from a cryotreated lesion was seen in five patients, three of whom died of the complication. One patient, who underwent cryosurgery for > 50% of liver volume, died of hepatic failure. One patient, who received cryosurgery for eight large metastases, died of a cryoshock syndrome. Three patients suffered from biliary fistula which resolved with transhepatic drainage. Five patients had temporary renal insufficiency, which presented as increased blood urea nitrogen and creatinine levels for 3-7 d. Two patients developed bacterial hepatic abscess within cryotreated sites and recovered with antibacterial agents and drainage. Two patients, aged 72 and 76 years respectively, died of acute myocardial infarction and severe arrhythmias apparently unrelated to cryosurgery. There were a total of seven patients who died from the main adverse effects after cryosurgery.

DISCUSSION

Cryosurgery is a treatment in which tumors are frozen and then left *in situ* to be reabsorbed. Several studies have reported the results of hepatic cryosurgery for treatment of hepatic colorectal metastases^[13-16]. Survival after

Table 6 Results of hepatic cryosurgery for colorectal metastases

Authors	No. of cases	Mode of cryo	Operative mortality (%)	Associated therapy (No. of patients)	Follow-up (mo)	Median survival (mo)	Survival (%)			
							1 yr	2 yr	3 yr	5 yr
Korpan 1997 ^[22]	63	OC	0	Resection	6-120				60	44
Wallace 1999 ^[23]	137	OC	0	Resection	14 (1-60)	23	86	47	29	
Weaver 1995 ^[15]	47	OC	4	Chemo	26 (24-57)			62		
Ruers 2001 ^[21]	30	OC		Resection	26 (9-73)	32	76	61		
Cha 2001 ^[20]	21	OC		Resection	28 (18-51)				70 ¹	
Bilchik 2001 ^[19]	153	OC		Chemo		28				
Goering 2002 ^[18]	42	OC		Resection		45	82		55	39
Kerkar 2004 ^[14]	98 ²	OC			54 (9-98)	33	81	62	48	28
Jungrai Thmayr 2005	17	OC	0		23 (2-65)	21	52	36	10	5
This study 2007	326	PC	0	TACE	32 (7-62)	29	78	62	41	23

¹Thirty mo survival; ²Including 56 cases of hepatic colorectal metastases. Cryo: Cryosurgery; OC: Operative cryosurgery; PC: Percutaneous cryosurgery.

cryosurgery is probably inferior to that achieved by liver resection, but it should be noted that most of the patients undergoing cryosurgery have non-resectable tumors or later stages of the disease. Current long-term follow-up has shown that cryosurgery is an important option for a wide range of non-resectable hepatic colorectal metastases and provides the potential for long-term survival^[17].

Until now, cryosurgery for most patients with hepatic colorectal metastases was performed during laparotomy, either as a single modality or in association with liver resection. Operative cryosurgery is still more invasive for the patient. As an advancement of imaging guidance and improvement of cryosurgical apparatus, the percutaneous mode of cryosurgery, a less invasive procedure, has been used for treatment of tumors, and apparently, may be suitable for non-resectable hepatic colorectal metastases.

Efficacy of percutaneous cryosurgery

This study, in which 326 patients with non-resectable hepatic colorectal metastases who underwent percutaneous cryosurgery were followed-up for a median of 36 mo, showed: (1) after cryosurgery, serum CEA in 77.5% of patients with elevated markers returned to normal. (2) After cryosurgery, CR was achieved in 14.6% of patients, PR in 41.1%, and SD in 24.3%. (3) During a median follow-up of 36 mo (7-62 mo), the median survival of all patients was 29 mo (range 3-62 mo). Overall survival was 78%, 62%, 41%, 34% and 23% at 1, 2, 3, 4 and 5 years, respectively.

Comparing the published results of operative cryosurgery (Table 6), in which the median survival was 21-45 mo, and the 1-, 2-, 3- and 5-years survival was 52%-86%, 36%-62%, 10%-70% and 5%-44%, our results are encouraging, especially in terms of non-resectable tumors.

Similar to operative cryosurgery, the main problem in the face of percutaneous cryosurgery is recurrence. In our patients, 47.2% had recurrence during a median follow-up of 32 mo (range, 7-61). The liver was the main site of recurrence, 61% of which occurred in the liver only, and 13.9% in the liver and some extrahepatic locations. Extrahepatic recurrence was mainly seen in the lungs and lymph nodes. The overall recurrence rate was lower than the 44% in a mean follow-up of 16 mo, reported by Adam^[13], and much lower than the 78%

reported by Weaver^[16]. It is important to point out that in our patients, the recurrence rate at cryotreated site, including at cryotreated site only as well as both of cryotreated site and the remaining areas of liver, was only 15.3% for patients with recurrence and 6.4% for all cases, which is significantly lower than the 58.8% reported by Jungrai Thmayr^[24]. Obviously, the decreased tumor recurrence is related to better survival.

Factors influencing survival of patient

This study showed patients with lesions ≤ 3 cm had an increased survival rate compared with those with lesions > 3 cm, with a median survival of 39 and 21 mo, respectively. This may have been due to the larger tumor in the vicinity of large vessels and exposure to the heat sink effect. The warming effect of blood flow can cause insufficient cryodestruction of the tumor. Pearson *et al*^[25] have reported that 66.7% of local recurrence occurs directly in the vicinity of the vena cava or a large vessel. No significant correlation has been found between the number of metastases and survival. Patients with tumor in the right hepatic lobe have higher survival compared with those with left lobe or bilateral tumors, which may be because the latter location is closer to large vessels.

This study also appeared to show a correlation between poor survival and CEA level > 100 ng/dL, with a median survival of 18 mo, which is much less than the 38 mo median survival for patients with lower CEA ($P < 0.01$). The result is consistent with that reported by Weaver *et al*^[15,16] who showed that patients with CEA > 100 ng/dL prior to cryosurgery had only 10 mo median survival, while the median survival of patients with CEA lower than that level was 17-19 mo. The poor outcome of patients with higher CEA may be related to the biological behavior of CEA-secreting tumors.

According to the results of this study, the possibility of repeat percutaneous cryosurgery may be a factor which brings about better survival and low recurrence. Patients who received two or three cryosurgery procedures had longer survival. In contrast to operative cryosurgery, percutaneous cryosurgery may be performed many times because of its convenience and low intervention. As a result, the recurrence in liver and extrahepatic metastases may be conveniently treated. In this series, there were 12 and 6 patients, respectively, with lung and pancreas

metastases, who were treated by percutaneous cryosurgery.

As shown in this study, patients who received post-cryosurgery TACE had the higher survival compared to patients who did not receive TACE (5-year survival of 47% *vs* 15%). Post-resection TACE has been shown to decrease the recurrence rate for patients with HCC^[26]. The effect, therefore, may be an additional factor for the longer survival in our patients. We have used combination of percutaneous cryosurgery and absolute ethanol injection for treatment of HCC with good results^[27]. The strategy may be suitable for hepatic colorectal metastases.

Safety of percutaneous cryosurgery

In this study, a total of 526 procedures of cryosurgery in all patients were safely performed percutaneously. There were 151 patients who underwent repeat cryosurgery, as many as two to four procedures, for recurrent tumors in the liver and extrahepatic loci. There was no treatment-related mortality. Although about one third of patients had adverse effects, such as pain, fever, increased liver enzymes, thrombocytopenia and pleural effusion, they were generally self-limited without poor outcome. Cryoshock, as the most serious complication of hepatic cryosurgery, was observed in one of our patients, with an incidence of 0.3%, which is lower than the 1% in patients who underwent operative hepatic cryosurgery, based on a worldwide survey^[28]. Other major adverse effects, such as hepatic bleeding, biliary fistula, liver failure, renal insufficiency and liver abscess, were observed in 0.3%-1.5% of our patients; however, the incidence is no higher than that in patients undergoing operative hepatic cryosurgery^[29,30]. Therefore, percutaneous cryosurgery is a safe technique for liver surgery.

In conclusion, the results of this study clearly show that percutaneous cryosurgery is a safe modality for hepatic colorectal metastases. Rather than an alternative to resection, the technique should be regarded as a complement to hepatectomy and as an additional means to achieve tumor eradication when total excision can not be accomplished.

COMMENTS

Background

Nearly 50% of patients who have colorectal carcinoma will develop liver metastases, which are a frequent cause of death. Without treatment, the average survival of patients with multiple lesions is 5-10 mo, and the 5 years survival rate is 1%. Liver resection is the only established, potentially curative treatment for patients with hepatic colorectal metastases confined to the liver, however, most patients have non-resectable tumors, even when they have no extrahepatic disease. If patients have more than three lesions or have anatomical constraints at presentation, only 5%-10% are suitable for liver resection. Palliative resection is of no significant benefit. Chemotherapy, including intra-arterial continuous infusion, is the most commonly used therapeutic modality but prolongation of survival has not been proven. Irradiation of liver metastases has been shown to relieve pain, but median survival does not appear to be prolonged. Therefore, the search for new treatment modalities for patients with non-resectable hepatic colorectal metastases is very urgent.

Research frontiers

Cryosurgery has increasingly been recognized as a safe and effective alternative treatment modality that is used alone or in conjunction with hepatic resection for a selected group of patients with non-resectable disease confined to the liver. It has shown some promising results, with a median survival > 2 years in most published studies and the prospect of long-term disease-free survival or cure for some patients.

Innovation and breakthroughs

Cryosurgery in most patients with hepatic colorectal metastases has been performed with open laparotomy. With improved imaging techniques and cryosurgical equipment, percutaneous cryosurgery, as a minimally invasive alternative for open procedure, is increasingly used for treatment of liver tumors. This study of 326 patients with hepatic colorectal metastases who underwent percutaneous cryosurgery demonstrates encouraging efficacy, with overall survival of 78%, 62%, 41%, 34% and 23% at 1, 2, 3, 4 and 5 years, respectively. These survival rates are much greater than those achieved using other treatments in patients with non-resectable liver metastases from colorectal carcinoma. Moreover, the incidence of complications is no higher than that in patients who underwent operative hepatic cryosurgery.

Applications

Percutaneous cryosurgery is a safe and feasible approach for treatment of patients with non-resectable hepatic colorectal metastases. Rather than an alternative to resection, it is to be regarded as a complement to hepatectomy and as an additional means to achieve tumor eradication when total excision can not be accomplished.

Terminology

Ablation destroys tumors locally without requiring resection, with effectiveness comparable to resection. Cryosurgery is one of the ablation techniques. Cryosurgery is referred to as cryotherapy or cryoablation, and is the *in situ* destruction of undesirable tissue by localized freezing. It has become an effective technique for treating tumors of solid organs such as liver, prostate, kidneys, lungs, and has advantages over other surgical techniques. Percutaneous cryosurgery is a minimally invasive technique, which is usually performed by the insertion of one or more cryosurgical probes into the tumor under guidance of ultrasound, CT or MRI.

Peer review

This is a valuable paper, which describes the efficacy and safety profile of percutaneous cryosurgery. A prospective trial that compares surgical cryoablation and other forms of ablation in terms of patient survival is necessary to confirm the effects of ablation for non-resectable liver cancer.

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Activator protein-1 involved in growth inhibition by RASSF1A gene in the human gastric carcinoma cell line SGC7901

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Abstract

AIM: To investigate the role of Ras association domain family protein 1 isoform A (RASSF1A) in gastric tumorigenesis.

METHODS: Through over-expression of RASSF1A gene in the SGC7901 cell line which was induced by a lipofectamine-mediated gene transfer approach. Activator protein-1 (AP-1) DNA binding activity was measured by electrophoretic mobility shift assay (EMSA).

RESULTS: Compared with the control clones, cells over-expressing RASSF1A exhibited significant inhibition of cell growth with G₁ cell cycle arrest *in vitro* and *in vivo*. The over-expression of RASSF1A significantly inhibited AP-1 activity in SGC7901 cells (0.981 ± 0.011 vs 0.354 ± 0.053 , $P < 0.001$). In addition, both Western blot analysis and immunocytochemistry demonstrated that RASSF1A down-regulated the expression of c-Fos (0.975 ± 0.02 vs 0.095 ± 0.024 , $P < 0.001$) but not c-Jun.

CONCLUSION: Over-expression of RASSF1A inhibits the growth of SGC7901 cells by negatively regulating the AP-1 activity, the latter in turn negatively signals cell proliferation.

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Key words: RASSF1A; Gastric adenocarcinoma; SGC7901; Activator protein-1

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INTRODUCTION

Gastric carcinoma is one of the most frequent tumors that seriously threaten people's health in China^[1]. Molecular genetics studies indicate that loss of 3p was observed in different types of solid tumors^[2]. Frequent loss on 3p21-23 was detected in gastric cancer^[3,4].

Ras association domain family protein 1 isoform A (RASSF1A), one transcript of RASSF1 gene, is a recently identified 3p21.3 tumor suppressor gene^[5]. Loss of expression of RASSF1A was a frequent event in primary gastric carcinoma^[6,7]. However, the exact role of RASSF1A in gastric tumorigenicity is largely unknown.

Activator protein-1 (AP-1) plays an important role in various human diseases and regulates the expression of multiple genes essential for cell proliferation, differentiation and apoptosis^[8]. AP-1 is thought to serve as a nuclear target of Ras^[9]. It is not known whether RASSF1A, as effectors of Ras signaling^[10], could or could not inhibit the activity of AP-1.

In this study, we established gastric cancer cell lines stably over expressing RASSF1A. Characterization of these cells with regard to proliferation rate and tumorigenicity *in vitro* and *in vivo* was performed. AP-1 activity was measured by electrophoretic mobility shift assay (EMSA). Our results suggest that over-expression of RASSF1A exerts inhibitory effects on the transformed phenotype of gastric cancer cells and RASSF1A inhibits AP-1 activity.

MATERIALS AND METHODS

Cell culture

The human gastric cancer cells, SGC7901 (Shanghai Cell Bank, Chinese Academy of Sciences), were maintained in RPMI 1640 medium (Life Technologies, Inc, Grand Island,

NY) supplemented with 100 mL/L fetal bovine serum plus penicillin (50 IU/mL) and streptomycin (50 µg/mL) with passage every three days. Cultures were incubated in an incubator containing 5 mL/L CO₂ at 37°C.

Gene transfection and establishment of stable cell lines

Plasmid pcDNA3.0-RASSF1A and pcDNA3.0 were gifts from Professor Michael White (Department of Cell Biology, UT Southwestern Medical Center, Dallas, TX 75390, USA). Cells were seeded in six-well plates to 70%-80% confluence. The cells were transfected with 4 µg/well plasmids using Lipofectamine 2000 (Invitrogen). After transfection for 6 h, the cells were transferred to normal medium and allowed to recover overnight. The cells were trypsinized and split 1:10, and then seeded into new six-well plates. 48 h after transfection, transfected cells were grown in RPMI containing G418 (Alexis Biochemicals) at 0.8 g/L until all of the nontransfected cells were dead (2 wk). Resistant clones were selected separately using cloning cylinders and maintained in RPMI containing 0.2 g/L G418 for further study. Meanwhile, SGC-7901 cells were transfected with the empty pcDNA3.0 vector as the control.

Preparation of cytoplasmic and nuclear extract

Nuclear and cytoplasm extracts were prepared as described by Dignam *et al*^[11]. Confluent cells in 10 cm dishes were treated for various times with the indicated effectors. Cells were resuspended in 400 µL of buffer A [10 mmol/L HEPES (pH 7.9), 1.5 mmol/L MgCl₂, 10 mmol/L KCl, 0.5 mmol/L DTT, 0.5 mmol/L phenylmethylsulfonyl fluoride, 1 µg/mL leupeptin, 1 µg/mL aprotinin, and 1 µg/mL pepstatin A], kept on ice for 15 min, lysed gently with 12.5 µL of 10% NP40, and centrifuged at 2000 r/min for 10 min at 4°C. The supernatant was collected and used as the cytoplasm extracts. The nuclei pellet was resuspended in 40 µL of buffer C [20 mmol/L HEPES (pH 7.9)], containing 1.5 mmol/L MgCl₂, 450 mmol/L NaCl, 25% glycerol, 0.2 mmol/L EDTA, 0.5 mmol DTT, 0.5 mmol/L phenylmethylsulfonyl fluoride, 1 µg/mL leupeptin, 1 µg/mL aprotinin, and 1 µg/mL pepstatin A] and agitated for 30 min at 4°C, and the nuclear debris was spun down at 20 000 r/min for 15 min. The supernatant (nuclear extract) was collected and stored at -80°C until ready for analysis. Proteins were measured using the BCA kit (Pierce) according to the manufacturer's protocol.

Western blot analysis

Eighty µg of cytoplasm proteins or 40 µg nuclear proteins were separated by 10% SDS-PAGE under reducing conditions, and transferred to a nitrocellulose membrane. The nitrocellulose membrane was then incubated with blocking buffer (TBST containing 5% non-fat milk) for 2 h at room temperature and with mouse monoclonal antibody against RASSF1A (Abcam, USA), c-Jun, c-Fos, CyclinD₁ (Santa Cruz Biotechnology, USA) overnight at 4°C with gentle shaking. The membrane was washed with TBST twice for 5 min, and then incubated with rabbit anti-mouse IgG conjugated horseradish peroxidase diluted at 1:2000 (Santa Cruz Biotechnology, USA) for 2 h at room temperature. After washing, RASSF1A was detected using

DAB reagents. The level of β-actin or tubulin was used as a control for equal loading of protein.

Reverse transcription-PCR analysis

Total RNA from SGC7901 cells was obtained using a RNA Mini Kit (Qiagen, Inc). Two µg of total RNA extracted from each cell line were reverse-transcribed using a RevertAid First Strand cDNA Synthesis Kit (MBI). Five ng of reverse-transcribed cDNA per sample were used to perform PCR in triplicate samples for RASSF1A and β-actin as an internal control. Reactions were carried out under the following conditions: 95°C for 5 min, followed by 35 cycles at 94°C for 30 s, and 52°C for 40 s and 72°C for 60 s. The following primers were used: RASSF1A forward, 5'-TCTGGGGCGTCGTGAGTAAA-3' reverse, 5'-CCACCACCAAGAACAGTCG-3', β-actin forward, 5'-CCTTCCTGGGCATGGAGTCCT-3', β-actin reverse 5'-GGAGCAATGATCTTGATCTT-3'. Three independent measurements were averaged and relative gene expression levels were calculated as a ratio to β-actin expression of each.

MTT assay

Cells were cultured in 96-well microtiter plates at a density of 1×10^4 cells per well. The surviving cells were measured by MTT assay at 1 d, 2 d, 3 d, 4 d, 5 d, 6 d, 7 d after seeding. 20 µL of 5 g/L MTT [3-(4,5-dimethyl-thiazolyl-2)-2,5-diphenyltetrazolium bromide, Fluka, Buchs, Switzerland] in PBS was added to each well and the cells were incubated for another 4 h at 37°C. The supernatant was removed, and 150 µL of DMSO was added to each well. The absorbency at a wavelength of 595 nm was measured with a micro ELISA reader (BioRad, CA, USA).

Flow cytometry analysis

Cells were collected and fixed in 70% of ice-cold ethanol in phosphate buffer saline (PBS) and stored at -20°C. After resuspension, 100 µL RNAase I (1 g/L) and 100 µL propidium iodide (PI, 400 g/L, Sigma, USA) were added and incubated at 37°C for 30 min. Analysis of samples was performed by flow cytometry (Coulter Epics, XL, UK). The cell cycle phase distribution was calculated from the resultant DNA histogram using Multicycle AV software (Phoenix Flow System, San Diego, CA, USA).

Plating efficiency

Plating Efficiency was prepared as described by Hu *et al*^[12]. Cells (1×10^2) were plated in 6-well plates. Colonies were scored at 14 d, fixed with 70% ethanol, stained with 5% Giemsa (Sigma), and counted under a microscope. Only those colonies containing at least 50 cells were considered to be viable survivors. The plating efficiency (PE) was calculated as follows: PE = (colonies formed/cells seeded) × 100%.

Tumorigenicity in nude mice

Single cell suspensions were trypsinized and collected. The cell viability was > 95% as determined by trypan blue staining. Cells (5×10^6) in a 0.1 mL volume of RPMI were inoculated s.c. into the right flank of 4-6 wk-old female BALB/c-nu/nu mice (Laboratory Animal Unit, Central

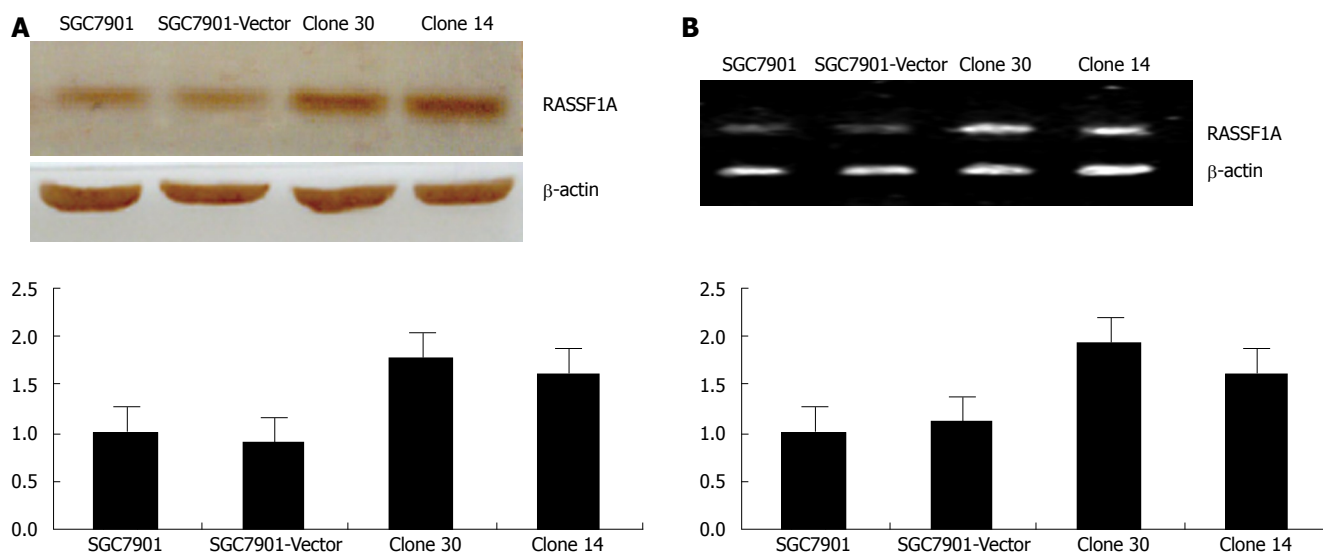


Figure 1 The over-expression of RASSF1A in gastric cancer cell line SGC7901. Cells were stably transfected using Lipofectamine 2000 with RASSF1A or empty vector and were grown in RPMI containing G418 at 0.8 g/L. Resistant clones were selected separately and were measured by Western blotting and RT-PCR, β -actin was used as an internal loading control, and clone30 was used for further study. **A:** RASSF1A expression was analyzed by Western blotting; **B:** RASSF1A expression was analyzed by RT-PCR.

South University). The mice were maintained under sterile conditions for 30 d. At the end of the experiment, the tumors were excised and the tumor weight was measured.

Electrophoretic mobility shift assay

EMSA was prepared as described by Li *et al*^[13]. Fifteen μ g of nuclear proteins were incubated with 1 μ g each of poly (dI-dC) in the presence of 30 fmol of digoxin (DIG)-labeled double-stranded AP-1 probe (5'-CGCTTGATG ACTCAGCCGGAA-3', BoYa Biotechnology, China) for 15 min at room temperature in a total volume of 20 μ L using DIG gel shift kit (Roche Diagnostics GmbH, Mannheim, Germany). Oligonucleotide competition experiments were performed in 50-fold excess of unlabeled oligonucleotides. DNA complexes were resolved from free probe with 4% nondenaturing polyacrylamide gels in 0.5 \times Tris-borate-EDTA (pH 8.3) and visualized by fluorography.

Immunocytochemistry

Cells were grown to 70% confluency on 22 mm \times 22 mm microscope coverslips, washed with PBS, then incubated for 1 h at room temperature with c-Jun, c-Fos monoclonal antibody (Santa Cruz Biotechnology, USA) at a final dilution of 1:200. Primary antibody was removed by repetitive washes with PBS and secondary antibody was added for 1 h at room temperature. Cells were washed in PBS and stained with DAB.

Statistical analysis

The data shown were mean values of at least three different experiments and expressed as mean \pm SD. Student's *t* test was used for comparison. $P < 0.05$ is considered statistically significant.

RESULTS

Generation of gastric cancer cells stably over-expressing RASSF1A

SGC-7901 human gastric cancer cells were transfected

with the control pcDNA3.0, pcDNA3.0-RASSF1A plasmids, respectively. Empty vector pcDNA3.0-transfected cell clones were named vector control and, together with the parental SGC-7901 cells, served as controls in this study. After G418 selection, 4 clones of pcDNA3.0-RASSF1A cells were picked, spread, and collected, including clone14, clone 19, clone 30 and clone 33. The expression of RASSF1A in clone 30 and clone 14 were further detected by Western blot analysis and RT-PCR analysis. As shown in Figure 1A, cells transfected with the control vector did not alter RASSF1A expression when compared with the SGC-7901 cells, whereas introduction of pcDNA3.0-RASSF1A resulted in marked over-expression. The protein expression of RASSF1A was increased about 77.5%, 61.2%, when compared with the parental cells. As shown in Figure 1B, RASSF1A mRNA was increased by 80.2%, 74.7%, respectively, in the corresponding cells. Clone 30, which showed the highest degree of over-expressing RASSF1A, consequently was selected for further study. Thus, the over-expression of RASSF1A was obviously increased in our established transfected cells.

Cell growth of SGC7901 cell inhibited by RASSF1A

To characterize the gastric cancer cells stably over-expressing RASSF1A protein, we first examined the possible effects on the rate of cell proliferation. Growth curves indicated that the vector control and parental cells displayed rapid growth rates, whereas the growth rate of the SGC7901-RASSF1A cells was significantly reduced (Figure 2A). The suppress rate was 28.13% at 48 h. The cell cycle distribution and apoptosis were determined by flow cytometry. As shown in Figure 2B, RASSF1A induced the cell cycle into G₁ phase. Compared with parental and vector control cells, the percentage of G₁ phase in RASSF1A transfected cells obviously increased ($P < 0.05$). And the apoptosis rate of cells expressing RASSF1A had a slight increase.

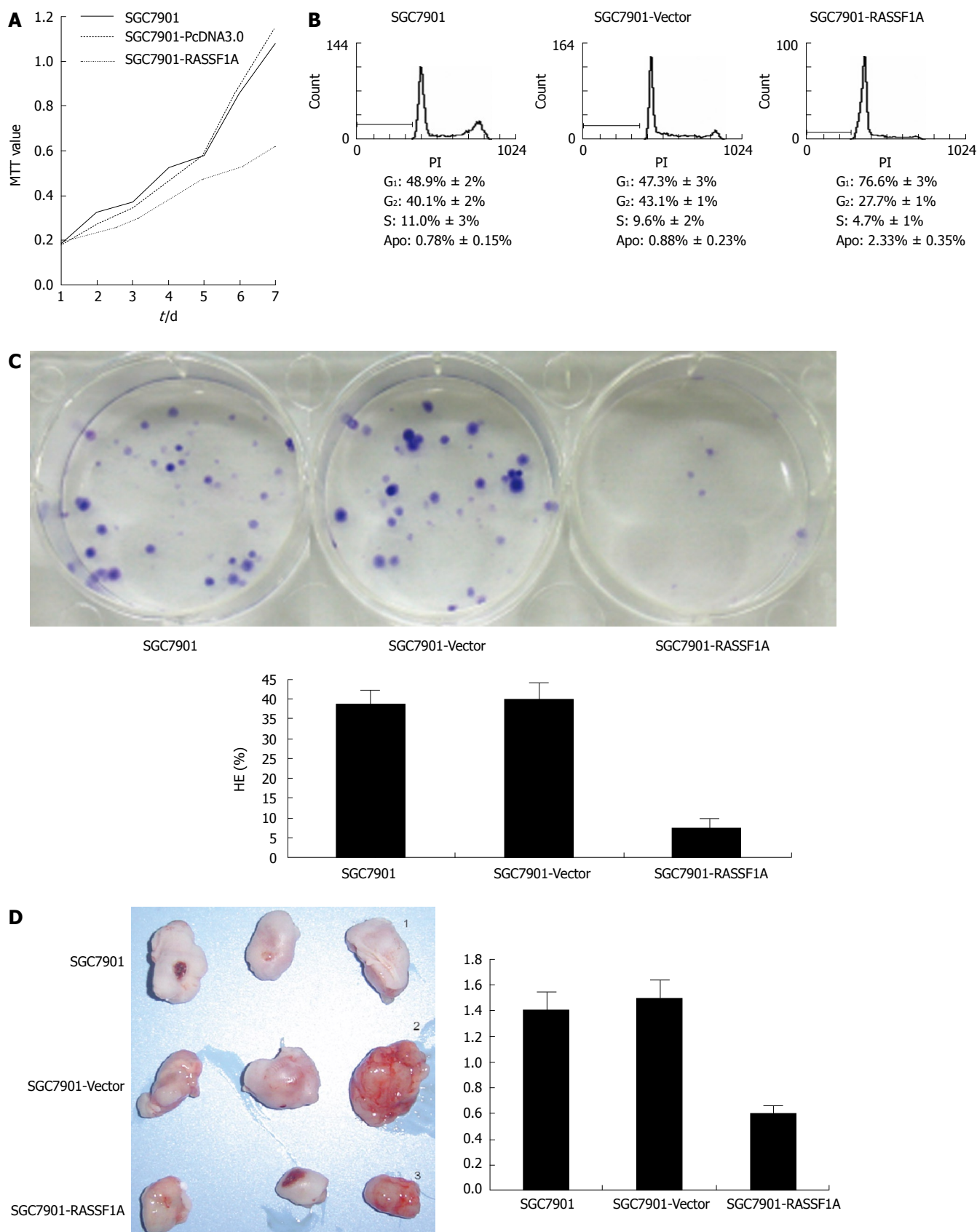


Figure 2 RASSF1A blocked gastric cancer cell line SGC7901 growth *in vivo* and *in vitro*. **A:** Cell growth curve of gastric cancer cell line SGC7901 measured by MTT assay; **B:** RASSF1A induced gastric cancer cell line SGC7901 G₁ arrest; **C:** RASSF1A inhibited the colony formation of SGC7901 cells measured by planting efficiency; **D:** RASSF1A inhibited the tumorigenicity of SGC7901 cells.

Colony formation and tumorigenicity inhibited by RASSF1A

We analyzed the colony forming ability of the RASSF1A

transfectants in planting because anchorage-independent growth often correlates with tumorigenicity. Planting efficiency in parent, vector control and RASSF1A

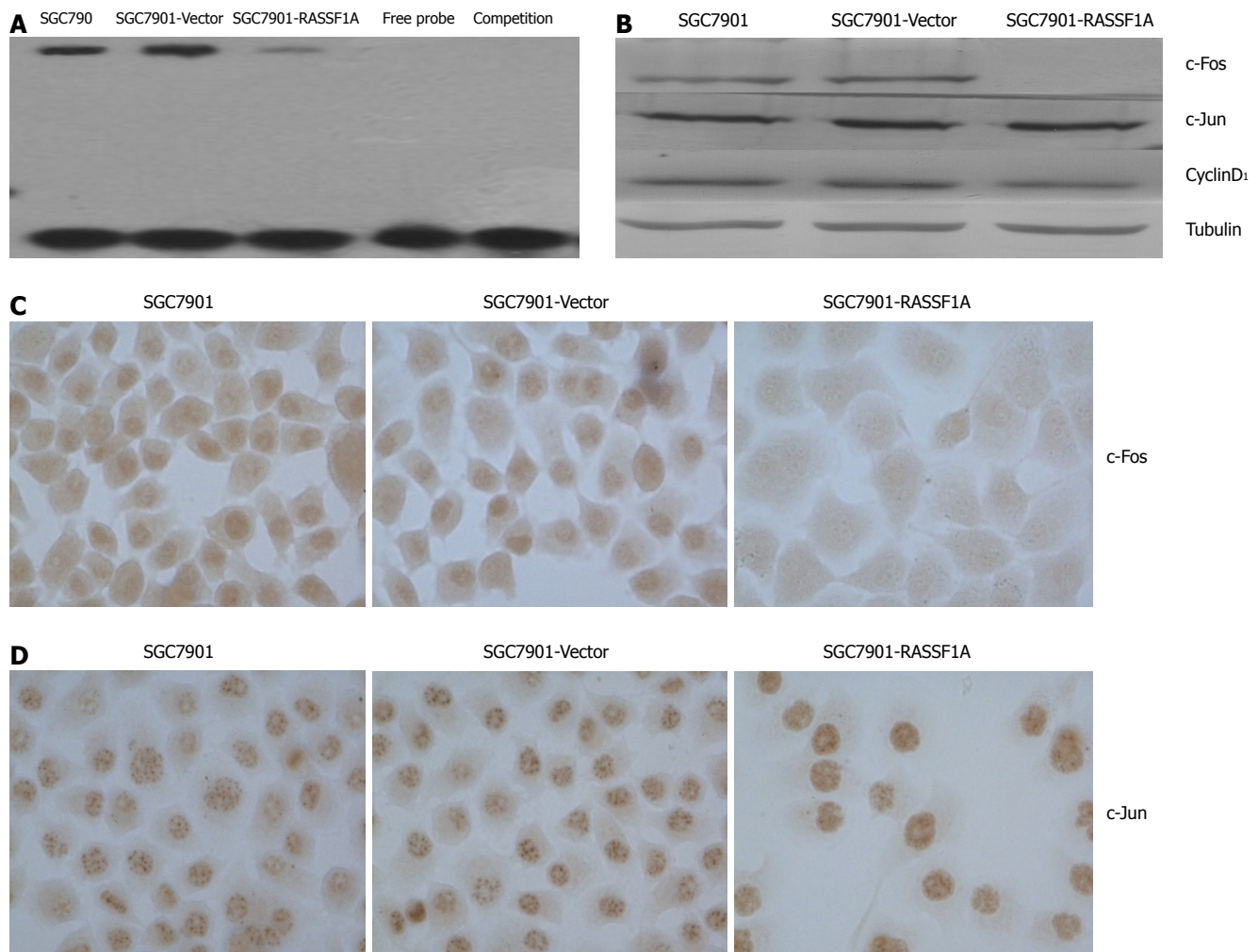


Figure 3 The over-expression of RASSF1A inhibited the AP-1 activity in gastric cancer cell line SGC7901. **A:** The over-expression of RASSF1A inhibited the AP-1 DNA binding activity measured by EMSA; **B:** The over-expression of RASSF1A inhibited the c-Fos but not c-Jun expression in nuclear extract of SGC7901 cells. (Western blotting, DAB staining); **C:** The over-expression of RASSF1A inhibited the c-Fos expression in nuclear of SGC7901 cells (immunocytochemistry, DAB staining, × 400); **D:** The over-expression of RASSF1A had no influence on the c-Jun expression in nuclear of SGC7901 cells (immunocytochemistry, DAB staining, × 400).

transfected cells were $38.6\% \pm 1.5\%$, $39.75\% \pm 2.1\%$ and $7.3\% \pm 0.6\%$, respectively (Figure 2C). SGC7901-RASSF1A cells displayed almost complete loss of colony-forming efficiency, and had a $> 80\%$ decrease when compared with the vector control and parental cells. In view of these results, we examined whether the RASSF1A over-expression in gastric cancer cells might affect their tumorigenicity in nude mice.

5×10^6 cells were injected s.c. into athymic nude mice and monitored for 30 d. The tumors appeared in three groups almost at the same time. At the end of the study, all of the tumors were removed and dissociated, and the weights of the tumors were measured. RASSF1A transfection revealed an obvious difference in tumor growth compared with vector control and parental cells during the observation period. The mean tumor weights in mice of parental, vector control cells and RASSF1A transfected cells were $1.4 \text{ g} \pm 0.26 \text{ g}$, $1.5 \pm 0.32 \text{ g}$ and $0.6 \pm 0.1 \text{ g}$, respectively (Figure 2D).

AP-1 activity inhibited by RASSF1A

We determined the DNA binding activity of AP-1 in SGC7901 cells by electrophoresis mobility shift assay.

The results showed that AP-1 DNA binding activity was significantly lower in SGC7901-RASSF1A cells than in vector control and parental cells (Figure 3A). Western blotting and immunocytochemistry data showed that c-Fos expression in the nuclear extract of SGC7901-RASSF1A cells was significantly inhibited, while, c-Jun expression in SGC7901-RASSF1A cells had no significant change. Furthermore, cyclinD₁ expression in nuclear extract was significantly lower in SGC7901-RASSF1A cells than in vector control and parental cells (Figure 3 B-D).

DISCUSSION

In this report, we have demonstrated that RASSF1A inhibited proliferation of SGC7901 cells. The cell growth was reduced by 28.13% at 48 h as determined by the MTT assay. The alteration of cell malignant phenotype was obvious as a result of loss of anchorage-independent growth ability as measured by a plating efficiency test. The tumorigenicity in nude mice was reduced significantly ($P < 0.01$). RASSF1A over-expression induced cell arrest from 48.9% to 76.6% ($P < 0.01$) in the G₁ population, and increased cell apoptosis rate from 0.78% to 2.33%

($P < 0.01$). These results are similar to the findings previously reported in other cancers, such as NSCLC^[5,10], kidney^[14], prostate^[15], nasopharyngeal carcinoma^[16], and glioma cell lines^[17]. RASSF1A differentially regulated many genes identified as having relevance to tumorigenesis including involvement in transcription, cytoskeleton, signaling, cell cycle, cell adhesion, and apoptosis^[18].

RASSF1A is predicted to encode a 39 kDa peptide that contains an N-terminal diacylglycerol (DAG)-binding domain, a Ras-association domain, a sequence PxxP and PEST sequences. The Ras-association domain is more than 50% identical and more than 70% similar to the carboxyl terminal 225 residues of mouse Nore1^[10]. RASSF1 binds Ras in a GTP-dependent manner, both *in vivo* and directly *in vitro*^[19]. It has also been shown to heterodimerize with Nore1^[20]. The presence of a Ras-association domain in both RASSF1 isoforms suggests that these proteins may function as effectors of Ras signaling (or signaling of a Ras-like molecule) in normal cells. The fact that RASSF1A can function as a tumor suppressor gene implies that RASSF1 acts in opposition to Ras-effector pathways stimulating proliferation^[10].

AP-1 is a transcription factor that consists of either a Jun-Jun homodimer or a Jun-Fos heterodimer. AP-1 regulates the expression of multiple genes essential for cell proliferation, differentiation and apoptosis^[8]. Our results showed that RASSF1A dramatically decreased basal AP-1 activity. We further detected the expression of c-Jun/c-Fos in SGC7901. Surprisingly we failed to observe any significant change of expression of c-Jun in the present study. However, expression of c-Fos had significant change. This indicated that RASSF1A could inhibit the expression of c-Fos but not c-Jun. However, in lung cancer cells, RASSF1A reduced c-Jun phosphorylation, suppresses the c-Jun-NH2-kinase pathway and inhibits cell cycle progression^[21]. We thought this difference may be due to the different histology and the role of c-Jun in RASSF1A-mediate growth inhibition in gastric carcinoma needs further investigation.

Shivakumar found that the exogenous expression of RASSF1A induced cell cycle arrest at the G₁ phase by down-regulating CyclinD₁^[22]. In agreement with this report, we also observed that the ectopic expression of RASSF1A down-regulated CyclinD₁. As a target of AP-1, CyclinD₁ plays an important role in cell proliferation^[23]. According to our results, we presumed that RASSF1A induced SGC7901 cell cycle arrest at the G₁ phase by down-regulating CyclinD₁ through inhibition of the activity of AP-1, but needs further investigation. Our current results indicate that inhibition of AP-1 activity contributes to RASSF1A mediated regulation of gastric carcinogenesis.

Thus, our data presented here clearly demonstrated that exogenous RASSF1A inhibits the growth of gastric carcinoma cells SGC7901 and RASSF1A gene may be a suppressor in gastric carcinogenesis. AP-1 may be involved in growth inhibition by the RASSF1A gene in the human gastric carcinoma cell line SGC7901.

of RASSF1 gene, is a recently identified 3p21.3 tumor suppressor gene and it is described as an effector of Ras signaling. Expression loss of RASSF1A was a frequent event in primary gastric carcinoma. However, the exact role of RASSF1A in gastric tumorigenicity is largely unknown.

Research frontiers

Gastric carcinoma is one of the most frequent tumors that seriously threaten people's health in China. Molecular genetics studies indicate that loss of 3p was observed in different types of solid tumors. Frequent loss on 3p21-23 was detected in gastric cancer. RASSF1A is predicted to encode a 39-kd peptide that contains an N-terminal diacylglycerol (DAG)-binding domain, a Ras-association domain, a sequence PxxP and PEST sequences. The presence of a Ras-association domain in both RASSF1 isoforms suggests that these proteins may function as effectors of Ras signaling (or signaling of a Ras-like molecule) in normal cells. The fact that RASSF1A can function as a tumor suppressor gene implies that RASSF1 acts in opposition to Ras-effector pathways stimulating proliferation.

Innovations and breakthroughs

Our study is believed to be the first to observe the role of RASSF1A in gastric cancer and the possible mechanisms involved.

Applications

Our research observed that RASSF1A suppressed the growth of the human gastric cancer cell line SGC7901 and RASSF1A inhibited AP-1 activity through down-regulated c-fos expression. This may have a significant clinical impact in the future.

Terminology

Electrophoretic mobility shift assay (EMSA): The EMSA technique is based on the observation that protein: DNA complexes migrate more slowly than free DNA molecules when subjected to non-denaturing polyacrylamide or agarose gel electrophoresis. Because the rate of DNA migration is shifted or retarded upon protein binding, the assay is also referred to as a gel shift or gel retardation assay.

Peer review

This is a well-written paper that suggests the role of RASSF1A in human gastric cancer. But the mechanism remains to be studied further.

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COMMENTS

Background

Ras association domain family protein 1, isoform A (RASSF1A), one transcript

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RAPID COMMUNICATION

Genetic polymorphisms in cytochrome P4502E1, alcohol and aldehyde dehydrogenases and the risk of esophageal squamous cell carcinoma in Gansu Chinese males

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Abstract

AIM: To evaluate the association between genetic polymorphisms in *CYP2E1*, *ALDH2* and *ADH1B* and the risk of esophageal squamous cell carcinoma (ESCC) in a high risk area of Gansu Province, in Chinese males.

METHODS: A case-control study was conducted to investigate the genetic polymorphisms of these enzymes (*CYP2E1**c1/*c2, *ALDH2**1/*2 and *ADH1B**1/*1 genotypes). A total of 80 esophageal cancer cases and 480 controls were recruited.

RESULTS: Compared with controls, cases had a greater prevalence of heavier alcohol consumption (53.8% vs 16.2%) and a higher proportion of alcohol drinkers with > 30 drink-years (28.8% vs 13.5%). Heavier alcohol consumption and alcohol drinking with > 30 drink-years increased the risk of ESCC, with ORs (95% CI) of 3.20 (1.32-9.65) and 1.68 (0.96-3.21). *CYP2E1* (*c1/*c1), *ALDH2* (*1/*2) and *ADH1B* (*1/*1) genotype frequencies were higher among patients with squamous cell carcinomas, at a level close to statistical significance ($P = 0.014$; $P = 0.094$; $P = 0.0001$ respectively). There were synergistic interactions among alcohol drinking and *ALDH2*, *ADH1B* and *CYP2E1* genotypes. The risk of the ESCC in moderate-to-heavy drinkers with an inactive *ALDH2* encoded by *ALDH2**1/*2 as well as *ADH1B* encoded by *ADH1B**1/*1 and *CYP2E1* encoded by *CYP2E1**c1/*c1 was higher than that in the never/rare-

to-light drinkers with an active *ALDH2* (*1/*1 genotype) as well as *ADH1B* (*1/*2 + *2/*2) and *CYP2E1* (*c1/*c2 + *c2/*c2) genotypes, with a statistically significant difference; ORs (95% CI) of 8.58 (3.28-22.68), 27.12 (8.52-70.19) and 7.64 (2.82-11.31) respectively. The risk of the ESCC in moderate-to-heavy drinkers with *ALDH2* (*1/*2) combined the *ADH1B* (*1/*1) genotype or *ALDH2* (*1/*2) combined the *CYP2E1* (*c1/*c1) genotype leads to synergistic interactions, higher than drinkers with *ALDH2* (*1/*1) + *ADH1B* (*1/*2 + *2/*2), *ALDH2* (*1/*1) + *CYP2E1* (*c1/*c2 + *c2/*c2) respectively, ORs (95% CI) of 7.46 (3.28-18.32) and 6.82 (1.44-9.76) respectively. Individuals with the *ADH1B* combined the *CYP2E1* genotype showed no synergistic interaction.

CONCLUSION: In our study, we found that alcohol consumption and polymorphisms in the *CYP2E1*, *ADH1B* and *ALDH2* genes are important risk factors for ESCC, and that there was a synergistic interaction among polymorphisms in the *CYP2E1*, *ALDH2* and *ADH1B* genes and heavy alcohol drinking, in Chinese males living in Gansu Province, China.

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Key words: Esophageal squamous cell carcinoma; Cytochromes P4502E1; Alcohol dehydrogenases; Aldehyde dehydrogenases; Genetic polymorphisms

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Guo YM, Wang Q, Liu YZ, Chen HM, Qi Z, Guo QH. Genetic polymorphisms in cytochrome P4502E1, alcohol and aldehyde dehydrogenases and the risk of esophageal squamous cell carcinoma in Gansu Chinese males. *World J Gastroenterol* 2008; 14(9): 1444-1449 Available from: URL: <http://www.wjgnet.com/1007-9327/14/1444.asp> DOI: <http://dx.doi.org/10.3748/wjg.14.1444>

INTRODUCTION

Esophageal carcinoma is the seventh leading cause of cancer deaths worldwide^[1]. It is a devastating disease with very few patients cured once diagnosed. Esophageal squamous cell carcinoma (ESCC) is one of the most

common malignancies in Gansu province, China. There is great geographic variation in the occurrence of this tumor type in China, including exceptionally high-risk areas such as Gansu Province in the Northwest of China. Within high-risk regions in China, there is a strong tendency toward alcohol drinking, suggesting that genetic susceptibility, in conjunction with alcohol consumption, plays a role in the etiology of ESCC.

Epidemiologic studies have demonstrated that drinking alcoholic beverages is causally related to the development of ESCC^[2,3]. Genetic polymorphisms in the genes encoding cytochrome P4502E1 (*CYP2E1*)^[4-7], aldehyde dehydrogenase-2 (*ALDH2*) and alcohol dehydrogenase-1B (*ADH1B*; previously called ADH2) affect the metabolism of alcohol^[8-12]. There have been some studies on the roles of alcohol and polymorphisms in the *CYP2E1*, *ALDH2* and *ADH2* genes in ESCC^[13-15]. However, their results were conflicting.

To provide further data on this issue, we evaluated the susceptibility to ESCC conferred by *CYP2E1*, *ALDH2* and *ADH1B* genetic polymorphisms, and defined the individual and combined roles of these genes and alcohol consumption in a high risk area for ESCC in Chinese males.

MATERIALS AND METHODS

The case participants in this study were 80 Gansu males with ESCC treated at the Department of Gastroenterology, First Hospital of Lanzhou University and the Department of Gastroenterology, Affiliated Hospital of Gansu College of Traditional Chinese Medicine. All were registered between September 2004 and March 2007.

The 480 age-and-gender matching controls consisted of cancer-free men who received annual health checkups at two Lanzhou clinics between September 2004 and March 2007. Gansu was the ancestral home for all.

Each participant independently completed a structured questionnaire concerning his alcohol drinking habits, and those with cancer were instructed to report their habits before they were diagnosed with cancer. Each participant was asked to classify himself as a drinker or non-drinker, and to report alcohol intake as the frequency of consumption and usual amount(s) and type(s) of alcoholic beverage(s) consumed. The subjects were classified as never drinks alcohol, or drinkers who consumed 200 g/wk (light drinkers), 200-400 g/wk (moderate drinkers) or 400+ g/wk (heavy drinkers).

Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) methods or a PCR method were performed on lymphocyte DNA samples from all participants, without any knowledge of their cancer status, to determine their P4502E1, *ALDH2* and *ADH1B* genotypes (Tables 1 and 2).

The Rsa I and Pst I linkage disequilibrium polymorphisms of the P4502E1 gene were determined according to the method of Hayashi *et al.*^[16] with some modification. The final mixture (50 μ L) was prepared containing 0.3 μ g of DNA, 0.2 μ mol/L of dNTP, 0.3 μ mol/L of each of the primers, 1.5 μ mol/L of MgCl₂, 5 μ L of 10 \times buffer, and 2 U of Taq polymerase. Briefly,

Table 1 Polymorphisms in the *CYP2E1*, *ALDH2* and *ADH1B* genes

Locus /protein	Gene	Subunit	Nucleotide change	Effect	RFLP
<i>ADH1B</i>	<i>ADH2*1</i> <i>ADH2*2</i>	β 1 β 2	48G>A	Wild-type His48; (earlier as His47)	
<i>ALDH2</i>	<i>ALDH2*1</i> <i>ALDH2*2</i>		1510G>A	Wild-type Lys487	
<i>CYP2E1</i>	<i>CYP2E1*1A</i> <i>CYP2E1*5B</i>		None -1293G>C; -1053C>T	Wild type	Pst I-/Rsa I + (c1allele) Pst I+/Rsa I - (c2 allele)

CYP2E1 allele nomenclature, <http://www.imm.ki.se>; NIAAA publications, <http://pubs.niaaa.nih.gov>.

Table 2 Primer sequences and lengths of PCR products

Gene	Primer	Size of PCR product (bp)	Chromosomal location
<i>CYP2E1</i>	5'-CCAGTCGAGTCTACATTGTC-3' 5'-TTCATTCTGCTCTTAACCTGG-3'	410	10q24.3-qter
<i>ALDH2</i>	5'-CCCTTTGGTGGCTAGAAGATG-3' 5'-CCACACTCACAGTTTCTCTT-3'	91	12q24.2
<i>ADH1B</i>	5'-ATTCTGTAGATGGTGGCTGT-3' 5'-GAAGGGGGTCACCAGGTG-3'	76	4q22

the samples were denatured at 94°C for 2 min and submitted to 40 cycles of 1 min at 94°C (denaturation), 50 s at 50°C (annealing) and 50 s at 72°C (extension), with a final extension at 72°C for 10 min. PCR products (15 μ L) were digested by Pst I or Rsa I restriction enzymes (1 μ L of a 10 U/ μ L preparation) for 18 h at 37°C. Fragments were separated on 40 g/L low melting point agarose gels, and stained with ethidium bromide.

ALDH2 and *ADH1B*^[17] polymorphisms were determined by PCR-RFLP as previously described. Each PCR analysis was performed twice, double blindly.

The allele frequency was determined by direct counting. Deviation of the genotype distribution from Hardy-Weinberg equilibrium was analyzed by the exact test. Fisher's exact test was used for comparing group statistics. The Spearman rank-correlation analysis was used as a nonparametric test for trend. All *P*-values were obtained from 2-sided tests. Associations between genotypes or other potential risk factors and ESCC are expressed as odds ratios (ORs) with 95% confidence intervals (CIs) adjusted for the effects of several possible confounders using a multiple logistic regression model and the STEPWISE method.

RESULTS

Five hundred and sixty males were enrolled in this study. The cancer cases were age-and-gender matched with cancer-free control subjects. The mean age of the patients was 60.2 \pm 8.9, ranging from 49 to 75 years of age. The mean age of the controls was 59.7 \pm 9.7, ranging from 49 to 73 years of age.

Table 3 Alcohol drinking in esophageal cancer cases and control subjects

Alcohol drinking	Cases (n = 80) %	Controls (n = 480) %	P	OR	95% CI
Status					
Never	4 (5.0)	132 (27.5)	0.17		
Former	46 (7.5)	36 (7.5)			
Current	20 (87.5)	312 (65.0)			
Dose					
Non-drinker	4 (5.0)	132 (27.5)	< 0.0001	1	
Light	10 (12.5)	153 (31.9)		0.16	0.03-0.24
Moderate	23 (28.7)	117 (24.4)		0.85	0.54-1.07
Heavy	43 (53.8)	78 (16.2)		3.20	1.32-9.65
Total years of drinking					
Never	40 (50.0)	243 (50.6)	0.009	1	
≤ 30	17 (21.2)	172 (35.8)		0.69	0.36-0.98
> 30	23 (28.8)	65 (13.5)		1.68	0.96-3.21

Table 4 Genotypes of *ALDH2*, *ADH1B* and *P4502E1*, n (%)

Genotype	Cases (n = 80)	Controls (n = 480)	P	OR	95% CI
<i>ALDH2</i> genotype					
1/1	37 (46.3)	252 (52.5)	0.094	1	
1/2	43 (53.7)	195 (40.6)		2.89	1.11-5.64
2/2	0 (0.0)	33 (6.9)			
<i>ADH1B</i> genotype					
1/1	17 (21.3)	24 (5.0)	< 0.0001	1	
1/2	25 (31.3)	168 (35.0)		3.67	1.26-8.73
2/2	38 (47.5)	288 (60.0)		1.46	0.71-2.59
<i>CYP2E1</i> Pst I/Rsa I					
c2/c2	7 (8.8)	75 (15.6)	0.918	1	
c1/c2	16 (20.0)	180 (37.5)			
c1/c1	57 (71.3)	225 (46.9)		2.82	1.23-6.55

Table 3 shows alcohol drinking in esophageal cancer cases and control subjects. We observed that, compared with controls, cases had a greater prevalence of heavier alcohol consumption (53.8% *vs* 16.2%) and a higher proportion of alcohol drinkers with > 30 drink-years (28.8% *vs* 13.5%). Heavier alcohol consumption and alcohol drinking with > 30 drink-years increased the risk of ESCC, with ORs (95% CI) of 3.20 (1.32-9.65) and 1.68 (0.96-3.21).

Table 4 shows the distributions of *ALDH2*, *ADH1B* and *CYP2E1* genotypes. Cases and controls differed significantly in the distributions of these genotypes. These genotypes significantly deviated from the Hardy-Weinberg equilibrium (HWE) in ESCC cases, but among controls, all genotypes were in HWE.

There are two *ALDH2* alleles (*1 and *2) and three genotypes: *1/*1 (GG, typical homozygote), *1/*2 (GA, heterozygote) and *2/*2 (AA, atypical homozygote), and the distributions of these genotypes were significantly different between the esophageal cancer group and the control group ($\chi^2 = 2.89$, $P < 0.1$). The prevalence of the inactive *ALDH2* encoded by *ALDH2**1/*2 was correlated with susceptibility to esophageal cancer, with an OR (95% CI) of 2.89 (1.11-5.64).

There are three *ADH1B* genotypes: The wild-type genotype (*1/*1), heterozygote (*1/*2) and homozygote (*2/*2) genotypes. The prevalence of the less-active

Table 5 Probability ratios for the combinations of *ALDH2*, *ADH1B* and *CYP2E1* genotypes and the amount of alcohol consumed, n (%)

Alcohol drinking	Genotype	Cases (n = 80)	Controls (n = 480)	OR (95% CI)	OR (95% CI)
<i>ALDH2</i>					
Never/rare	1/1	7 (8.8)	72 (15.0)	1	
-to-light	1/2	7 (8.8)	180 (37.5)	0.56 (0.20-1.59)	
	2/2	0 (0.0)	33 (6.9)	0.00 (NC)	
Moderate	1/1	30 (37.5)	180 (37.5)	2.29 (0.94-5.57)	
-to-heavy	1/2	36 (45.0)	15 (3.1)	8.58 (3.28-22.68)	3.12 (1.86-6.58)
<i>ADH1B</i>					
Never/rare	1/2+2/2	13 (16.3)	273 (56.9)	1	
-to-light	1/1	1 (1.3)	12 (2.5)	1.00 (0.18-9.22)	
Moderate	1/2+2/2	50 (62.5)	183 (38.1)	4.75 (2.53-9.38)	
-to-heavy	1/1	16 (20.0)	12 (2.5)	27.12 (8.52-70.19)	5.48 (1.98-14.55)
<i>CYP2E1</i>					
Never/rare		5 (6.3)	189 (39.4)	1	
-to-light	c1/c1	9 (11.3)	96 (20.0)	0.56 (0.20-1.59)	
Moderate	c1/c2+	18 (22.5)	96 (20.0)	1.93 (0.43-2.41)	
-to-heavy	c2/c2				
	c1/c1	48 (60.0)	99 (20.6)	7.64 (2.82-11.31)	5.32 (1.62-9.28)

Table 6 Probability ratios for the combinations of *ADH1B*, *CYP2E1* and *ALDH2* genotypes among moderate-to-heavy drinkers, n (%)

Combined genotypes	Genotype	Cases (n = 80)	Controls (n = 480)	OR	95% CI
<i>ALDH2</i> + <i>ADH1B</i>	<i>ALDH2</i> *1/1 + <i>ADH1B</i> *1/2 + 2/2	28 (35.0)	144 (30.0)	1	
	<i>ALDH2</i> *1/2 + <i>ADH1B</i> *1/1	15 (18.8)	27 (1.9)	7.46	3.28-18.32
	<i>ALDH2</i> *1/1 + <i>ADH1B</i> *1/1	10 (12.5)	60 (12.5)	1	
<i>ALDH2</i> + <i>CYP2E1</i>	<i>ALDH2</i> *1/1 + <i>CYP2E1</i> *c1/c2 + c2/c2	32 (40.0)	15 (3.1)	6.82	1.44-9.76
	<i>ALDH2</i> *1/2 + <i>CYP2E1</i> *c1/c1	12 (15.0)	36 (2.5)	1	
	<i>ADH1B</i> *1/2 + 2/2 + <i>CYP2E1</i> *c1/c2 + c2/c2	14 (17.5)	66 (13.8)	6	

ADH1B encoded by *ADH1B* *1/*1 increase the risk of esophageal cancer. ($\chi^2 = 18.664$, $P < 0.0001$), OR (95% CI) of 3.67 (1.26-8.73).

There are three *CYP2E1* genotypes: wild homozygote (*c1/*c1), heterozygote (*c1/*c2) and mutated homozygote (*c2/*c2) genotypes. A significant difference in the distributions of the three Pst I/Rsa I genotypes of *CYP2E1* was found between the esophageal cancer group and the control group ($\chi^2 = 5.977$, $P < 0.05$). The c1/c1 genotype was correlated with susceptibility to esophageal cancer, with an OR (95% CI) of 2.82 (1.23-6.55).

Tables 5 and 6 show the frequency distributions and ORs for each combination of alcohol drinking habits and *ALDH2*, *ADH1B* and *CYP2E1* genotypes.

The risk of ESCC was 8.58-fold higher in moderate-to-heavy drinkers with inactive *ALDH2* (encoded by

*ALDH2**1/*2) than in the never/rare-to-light drinkers with an active *ALDH2* (encoded by *ALDH2**1/*1).

When the ORs were compared within each alcohol drinking category, the risk of the ESCC associated with *ALDH2* (*1/*2) versus *ALDH2* (*1/*1) was 3.12-fold greater among moderate-to-heavy drinkers, whereas no significant increase in risk was observed with *ALDH2* *1/*2 versus *ALDH2* *1/*1 among never/rare-to-light drinkers.

The results for the *ADH1B* genotype showed that only among moderate-to-heavy drinkers did the less-active *ADH1B* *1/*1 genotype significantly increase the risk of cancer, with an OR (95% CI) 5.48 (1.98-14.55). Thus, the risk of the cancer in moderate-to-heavy drinkers with the *ADH1B* (1/1) genotype was markedly higher (by 27.12-fold) than that in never/rare-to-light drinkers with a super-active *ADH1B* encoded by *ADH1B* *1/*2 and *ADH1B* *2/*2.

The results for the *CYP2E1* genotype showed that, among moderate-to-heavy drinkers, the *CYP2E1* (*c1/*c1) genotype significantly increased the risk of cancer, with an OR (95% CI) of 5.32 (1.98-14.55). Thus, the risk of the cancer in moderate-to-heavy drinkers with *CYP2E1* (*c1/*c1) was markedly higher (by 7.64-fold) than that in never/rare-to-light drinkers with a super-active *CYP2E1* (genotypes *c1/*c2 + *c2/*c2).

The results of our analysis of *ALDH2* and *ADH1B* genotypes showed that among moderate-to-heavy drinkers, the combination of *ALDH2* (*1/*2) and *ADH1B* (*1/*1) genes synergistically increased the risk of cancer, to 7.46-fold higher than that in drinkers with an *ALDH2* (*1/*1) + *ADH1B* (*1/*2 + *2/*2) genotype.

The results of an analysis of the effects of combinations of *ALDH2* and *CYP2E1* genotypes showed that among moderate-to-heavy drinkers, the combination of *ALDH2* (*1/*2) and *CYP2E1* (*c1/*c1) genes synergistically increased the risk of cancer, to 6.82-fold higher than that in drinkers with an *ALDH2* (*1/*1) + *CYP2E1* (*c1/*c2 + *c2/*c2) genotype.

There was no combinatorial effect of *ADH1B* and *CYP2E1* genotypes.

DISCUSSION

In the present study, we examined the associations of ESCC with *ALDH2*, *ADH2* and *CYP2E1* genetic polymorphisms in conjunction with alcohol drinking habits among a population at high risk of esophageal cancer. The study was conducted in Gansu province, an area of China with a relatively high alcohol consumption rate.

We found that alcohol intake was associated with ESCC, and that polymorphisms in *CYP2E1*, as well as in the genes encoding alcohol and aldehyde dehydrogenases (*ADH1B* and *ALDH2*) are important risk factors for ESCC in Chinese men living in this high risk area.

We found that heavier alcohol consumption and alcohol drinking for > 30 drink-years could increase the risk of ESCC, with ORs (95% CI) of 3.20 (1.32-9.65) and 1.68 (0.96-3.21), respectively. There is substantial evidence that drinking alcohol increases the risk of ESCC. Alcohol can be considered to induce DNA damage and result in

the modification of nucleotides. Our risk estimates were consistent with those of previous studies^[18]. This study confirms that alcohol consumption contributes to the etiology of ESCC.

It has been reported that individual differences in cytochrome P450 (CYP) gene expression may contribute to a person's individual susceptibility to pro-carcinogens and, subsequently, to the development of malignancies. CYP plays a central role in the metabolism of carcinogens by activating oxidation reactions, and may be expressed in esophageal mucosa.

CYP2E1 metabolizes ethanol to acetaldehyde and is primarily responsible for the metabolic activation of many low molecular weight carcinogens, including certain nitrosamines, which may be involved in carcinogenesis of the esophagus. This enzyme is also believed to participate in the oxidation of other compounds, such as ethanol, to produce reactive free radicals that may initiate lipid peroxidation and consequently influence carcinogenesis. In addition, it effectively reduces dioxygen to give rise to radical species, thus contributing to lipid peroxidation and oxidative inhibition. Individuals with the variant Pst I/Rsa I allele (*c1/*c2 or *c2/*c2) have a lower basal *CYP2E1* activity. Two studies found an association between the *CYP2E1* Pst I /Rsa I variant allele and a decreased risk of ESCC^[19]. The results of our study indicated that the *CYP2E1* *c1/*c1 or c1 allele increased the susceptibility to ESCC in a Gansu population ($P = 0.014$), and that there are synergistic interactions between *CYP2E1* (*c1/*c1) and alcohol drinking; the risk of ESCC in moderate-to-heavy drinkers with the *CYP2E1* (*c1/*c1) genotype was markedly higher, by 7.64-fold, than in never/rare-to-light drinkers with a super-active *CYP2E1* (genotypes *c1/*c2 + *c2/*c2).

The genetic polymorphisms of alcohol and aldehyde dehydrogenases affect the metabolism of alcohol. Aldehyde dehydrogenase-2 (*ALDH2*) is the key enzyme for elimination of acetaldehyde. Acetaldehyde, a recognized animal carcinogen derived from alcohol, may play an important role in the pathogenesis of alcohol-related cancers, such as esophageal cancer. In persons with inactive *ALDH2* encoded by *ALDH2**1/*2, the body fails to metabolize acetaldehyde rapidly, leading to excessive accumulation of acetaldehyde.

Polymorphisms in the *ADH1B* and *ALDH2* genes associated with the risk of esophageal cancer have been described in several studies^[20-24]. *ADH1B* (*1/*1) has also been demonstrated to enhance the risk of esophageal cancer, and the combination of *ALDH2* (*1/*2) and *ADH1B* (*1/*1) increased the risk of esophageal cancer^[8].

In our study, we found that polymorphisms in the genes encoding alcohol and aldehyde dehydrogenases (*ADH1B* and *ALDH2*) are important risk factors for ESCC in a Gansu population, and individuals with the *ADH1B* (*1/*1) genotype had a 5.32-fold risk (1.98-14.55) of developing esophageal cancer compared with those with the *ADH1B* (*2/*2) genotype.

Individuals with the *ALDH2* (*1/*2) genotype had a 3.12-fold higher risk (1.86-6.58) of developing esophageal cancer than those with the *ALDH2* (*1/*1) genotype, among male Chinese moderate-to-heavy drinkers. We also

found that individuals with a combined *ALDH2* (*1/*2) and *ADH1B* (*1/*1) genotype showed a dramatically increased risk of ESCC, with an OR (95% CI) 7.46 (3.28-18.32), which is higher than those due to the respective genotypes. These findings indicate the *ALDH2* (*1/*2) genotype has synergistic interactions with the *ADH1B* (*1/*1) genotype, contributing to the development of ESCC. Our study confirmed the findings of Tetsuji^[8].

The significant finding in this study was the interaction between the *CYP2E1* and *ALDH2* genotypes and heavy alcohol drinking, using a case-control study design. Previous studies have not examined this issue in detail and, to our knowledge, this is the first study to show a significant interaction between the *CYP2E1* and *ALDH2* genotypes and alcohol drinking. In our study, we found synergistic interactions among polymorphisms in the *CYP2E1* and *ALDH2* genotypes and heavy alcohol drinking; individuals with a combined *ALDH2* (*1/*2) and *CYP2E1* (*c1/*c1) genotype showed a dramatically increased risk of ESCC, with an OR (95% CI) of 6.82 (1.44-9.76), which is higher than those due to the respective genotypes.

Conflicting results from studies^[25-28] in different countries and areas show the complexity of the biological mechanisms underlying ESCC. The susceptibility to ESCC may be correlated with genes, environment, area, race or other factors. The results of this study demonstrate that *CYP2E1* (*c1/*c1), *ALDH2* (*1/*2) and *ADH1B* (*1/*1) genotypes are associated with esophageal cancer risk among moderate-to-heavy drinking Chinese males in Gansu province. In addition, this study also showed *ALDH2* (*1/*2) and *CYP2E1* (*c1/*c1) carriers, and *ALDH2* (*1/*2) and *ADH1B* (*1/*1) carriers have a much higher risk of developing esophageal cancer, especially among alcohol drinkers. Future studies are needed to examine the biological mechanisms involved, and to evaluate the contribution of gene and environment interactions to the risk of ESCC.

COMMENTS

Background

Worldwide, cancer of the esophagus ranks among the 10 most common cancers. Epidemiological studies have demonstrated that drinking alcoholic beverages is causally related to the development of esophageal squamous cell carcinoma (ESCC). Genetic polymorphisms in the P4502E1 (*CYP2E1*), *ALDH2* and *ADH1B* genes affect the metabolism of alcohol. There have been some studies on the roles of alcohol and the *CYP2E1*, *ALDH2* and *ADH2* genes in ESCC. However, their results were conflicting. Therefore, the aim of the present study was to evaluate the susceptibility to ESCC conferred by *CYP2E1*, *ALDH2* and *ADH1B* genetic polymorphisms, and to define the individual and combined roles of these genes and alcohol consumption in a high risk area for ESCC, among Chinese males.

Research frontiers

Accumulating evidence from prior epidemiologic studies suggests an association between esophageal cancer and the use of alcohol. The genetic polymorphisms of alcohol and aldehyde dehydrogenases affect the metabolism of alcohol. *ALDH2* is the key enzyme involved in the elimination of acetaldehyde. Polymorphisms in the *ADH1B* and *ALDH2* genes are associated with the risk of esophageal cancer.

Innovations and breakthroughs

To our knowledge, this is the first study to show significant interactions among the *CYP2E1* and *ALDH2* genotypes and alcohol drinking. We found synergistic interactions among polymorphisms in the *CYP2E1* and *ALDH2* genes and heavy

alcohol drinking: Individuals with a combined *ALDH2* (*1/*2) and *CYP2E1* (*c1/*c1) genotype showed a dramatically increased risk of ESCC, which is higher than the risk of ESCC due to the respective genotypes.

Applications

The detection of *ALDH2*, *ADH1B* and *CYP2E1* genotypes may become a useful index for esophageal cancer, and also help clinicians to diagnose esophageal cancer earlier.

Terminology

Esophageal squamous cell carcinoma: The most common types of esophageal cancer are squamous cell carcinoma and adenocarcinoma. Squamous cell carcinoma develops in flat cells that line the esophagus. Approximately 60% of squamous cell carcinomas develop in the middle third of the organ, 30% occur in the lower third, and 10% occur in the upper third. Adenocarcinoma develops in the lining of the esophagus and is associated with a condition called Barrett's esophagus. This type usually occurs in the lower third of the esophagus. Genetic polymorphisms: The occurrence together in the same population of more than one allele or genetic marker at the same locus, with the least frequent allele or marker occurring more frequently than can be accounted for by mutation alone. Genetic polymorphisms provide us with the ability to predict inter-individual differences in susceptibility to clinical disease. Biomarkers of susceptibility include polymorphisms in carcinogen metabolism, DNA repair capacity and genes that control cell growth.

Peer review

This is an interesting study on the etiology of esophageal squamous cell cancer in China. They confirm a synergy between alcohol consumption and the phenotype of inactive enzymes.

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CASE REPORT

Cervical cellulitis and mediastinitis following esophageal perforation: A case report

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Abstract

Chicken bone is one of the most frequent foreign bodies (FB) associated with upper esophageal perforation. Upper digestive tract penetrating FB may lead to life threatening complications and requires prompt management. We present the case of a 52-year-old man who sustained an upper esophageal perforation associated with cervical cellulitis and mediastinitis. Following CT-scan evidence of FB penetrating the esophagus, the impacted FB was successfully extracted under rigid esophagoscopy. Direct suture was required to close the esophageal perforation. Cervical and mediastinal drainage were made immediately. Nasogastric tube decompression, broad-spectrum intravenous antibiotics, and parenteral hyperalimentation were administered for 10 d postoperatively. An esophagogram at d 10 revealed no leak at the repair site, and oral alimentation was successfully reinstituted. Conclusion: Rigid endoscope management of FB esophageal penetration is a simple, safe and effective procedure. Primary esophageal repair with drainage of all affected compartments are necessary to avoid life-threatening complications.

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Key words: Esophagus; Perforation; Foreign body; Mediastinitis; Surgery

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INTRODUCTION

Management of ingested foreign bodies (FB) is a common clinical encounter. Complications of this pathology are dependent on patient age, nature and localization of the FB, presence of a perforation, and initial management procedures^[1]. Beside the lingual tonsils, the base of tongue and the upper esophagus are the most common sites of FB impaction^[2]. The most frequent ingested FBs in the upper digestive tract are chicken and fish bones, and they are the most commonly associated with pharyngo-esophageal perforation^[1], cervical abscess and potentially life threatening complications^[3].

CASE REPORT

A 52-year-old man, with no relevant past medical history, presented to the ENT clinic complaining of severe dysphagia, substernal pain, and fever for 3 d. Five days prior to presentation, the patients started having symptoms of mild dysphagia after chewing on a piece of chicken. One day later on, exacerbation of dysphagia prompted the patient to seek otolaryngology consultation in town. Following seemingly normal head and neck examination, the patient was reassured and discharged. On admission at the University Medical center of Grenoble, the patient had a fever of 40°C and mild shortness of breath. No abnormalities were noted on examination of the oropharynx, but the hypopharynx and larynx were swollen and there was abundant saliva in hypopharynx. The neck exam revealed bilateral anterior and lateral tenderness, inflammation and edema of the skin, and crepitation. On pulmonary auscultation, sparse rales were heard over the lower part of each hemi-thorax. Cardiac auscultation revealed a regular rhythm and normal heart sounds.

The patient had a white blood cell count of $20 \times 10^9/L$ with 80% neutrophils and a C reactive protein level to 120 mg/L.

A cervical and thoracic CT-scan revealed a fragment of bone in the upper part of esophagus, air in the retropharyngeal space and the upper part of the posterior mediastinum, and deep subcutaneous collection suggestive of cervical posterior mediastinal collection (Figure 1).

The patient was taken immediately to the operating room for surgical treatment under general anesthesia. Endoscopic examination was performed using a rigid endoscope (Storz ϕ 16 mm - L 50 cm, Tuttlingen, Germany) which revealed meat fragments and a piece of chicken bone impacted in the upper esophageal wall, 19 cm

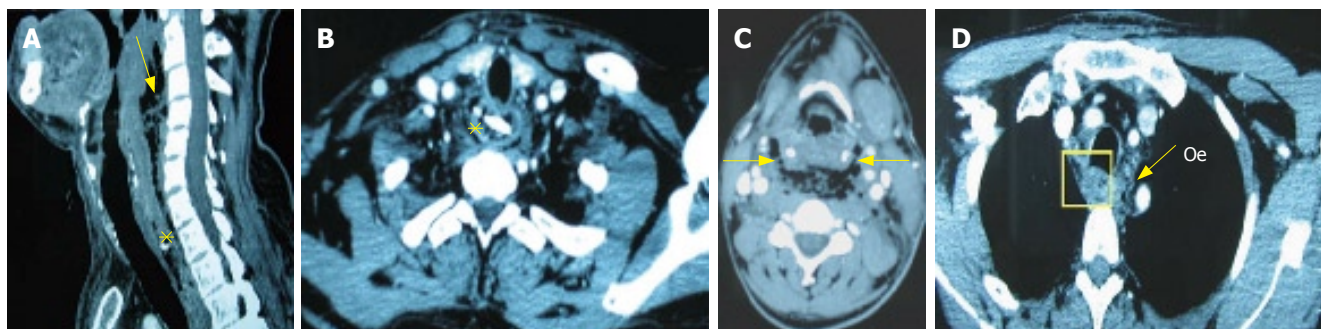


Figure 1 CT scan on admission. **A:** Mid-sagittal view: Air in posterior retropharyngeal space, Bone fragment (*); **B:** Axial view at the level of C7. Bone fragment and esophageal perforation (*); **C:** Axial view at the level of C4 (hyoid bone): Air in posterior retropharyngeal space; **D:** Axial view at the level of T3: Pleural effusion and air bubbles in postero-superior mediastinum → Oe: esophagus.

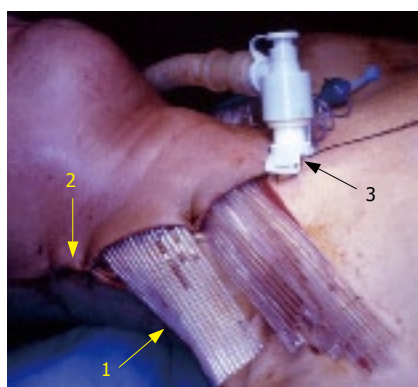


Figure 2 Surgical site at the end of the procedure. 1: Multitubular silicone drain; 2: Neck incision; 3: Tracheotomy tube.

from incisors. Meat fragments were aspirated using a 4 mm rigid aspiration; forceful pulling of the FB using forceps caused the edges of the FB to bend, enabling extraction through the rigid esophagoscope. After bone removal, a 4 mm perforation was visible at the site of impaction with pus draining through it. Gastric decompression *via* a nasogastric tube was performed.

A large neck incision was required to drain the pus from cervical spaces (Figure 2), and the collection in the upper posterior mediastinum was evacuated through the same incision following retropharyngeal space dissection. The drainage of the neck abscess revealed a small bone fragment within the purulent discharge, and cultures were taken. The edges of the perforation were carefully derided and the entire surgical field was well irrigated. The defect was then closed in two layers using fine interrupted absorbable monofilament suture. Drainage of the neck and the upper mediastinum was done by placement of multi-tubular silicone drains through cervical incision. A temporary tracheotomy was performed to bypass the edematous larynx. Broad-spectrum intravenous antibiotics (imipenem 2 g/d+ amikacine 1 g/d+ metronidazole 1 g/d), and parenteral hyperalimentation were started. The patient was admitted to the intensive care unit for 5 d. The patient medical status improved rapidly, and biological laboratory exams were normalized during the first post operative week. Tracheotomy and drains were removed at postoperative d 6 and d 8 respectively. An esophagogram

performed at d 10 revealed no leak at the repair site, and oral alimentation was successfully reinstituted. *Streptococcus* β C hemolytic was isolated from the cultured pus, allowing for switching to amoxicillin and clavulanic acid 4 g/d at postoperative d 3, and continued for 7 d. The patient was discharged from the hospital in good condition on postoperative d 15.

DISCUSSION

The majority of ingested FBs pass through the gastrointestinal tract uneventfully^[1]. Severe complications are rare, but upper digestive tract perforation is the most frequent. Among the esophageal perforation etiologies, FB represent the second most common etiology after iatrogenic manipulation (esophagoscopy, esophageal dilatation, para-esophageal surgery, external trauma)^[4]. The diagnosis of esophageal perforation is usually suspected on clinical basis, and suggestive history of sharp bodies ingestion (chicken and fish bones)^[5]. Symptoms include pain, dysphagia, and rarely hematemesis; pain is the most frequent symptom (> 90%)^[5]. In case of upper esophagus perforation, tenderness and subcutaneous emphysema of the neck are the two main signs. Substernal pain and polypnea are signs associated with mediastinal extension^[5]. In these cases, mediastinal crunch (Hamman sign) and crepitations are heard on chest auscultation^[6]. Fever and leukocytosis with increase in the number of immature polymorphonuclear cells are present in more than 90% of patients^[5].

Standard neck and chest radiograms are ordered if an esophageal perforation is suspected. But these exams reveal esophageal perforation in only a small proportion of cases^[7]. Moreover, even if FB is radiopaque, it can be unrecognized because a lot of bone structures are superposed. The perforation diagnosis is confirmed in almost all cases by contrast esophagograms, which can delineate both the level of perforation and the communication of the injury into cervical and mediastinal spaces^[6,7]. CT-scan allows the visualization of FB and the identification of findings suggestive of esophageal perforation (esophageal wall thickening and laceration, peri-esophageal air and fluid). Intravenous administration of contrast material helps CT-scan localization of the exact extension of peri-esophageal infection in the

neck, mediastinal, and pleuro-parenchymal spaces^[8]. Its sensitivity can be increased with gastrografen ingestion. Thus, an appropriate CT examination enables an accurate and timely diagnosis which significantly affects prognosis and provides valuable indications for treatment.

Extraction of FBs located in the first few centimeters under the upper esophageal sphincter, is difficult with a flexible esophagoscope, especially if they have sharp edges, such as bone fragments^[9]. Rigid endoscopy may be a more appropriate procedure in these instances. The rigid endoscope is placed just above the proximal tip of the FB; it dilates the esophageal lumen to the extent that the impacted FB is movable. The use of a rigid endoscope during removal of an impacted FB has several advantages: it causes expansion of the upper esophagus, which can release totally or in part the impacted FB, and prevents aspiration and esophageal or pharyngeal injury. It must be practiced under general anesthesia by a trained operator.

Non-operative management is excluded in case of cervical and/or mediastinal abscesses^[4]. Different surgical approaches are available for esophageal perforation, including primary repair with drainage, generally chosen for patients without evidence of a severe esophageal injury and without other esophageal disease^[10]. A simple suture is recommended in case of small perforation in the cervical esophagus^[5]. It is not necessary to use mucosal flaps to reinforce the esophageal sutures, contrarily to the recommendations for injuries of the middle and lower parts of esophagus^[11]. In case of cervical abscess and/or mediastinitis, drainage of the different affected spaces must be done^[12]. The choice of surgical approach for mediastinal drainage is dependent on abscess localization. In case of posterior and superior mediastinitis, drainage from cervical incision with retropharyngeal space dissection is adopted. Prognosis in case of upper esophageal perforation is relatively good with mortality inferior to 10%^[5].

In conclusion, perforation of the upper esophagus caused by a FB is rare, but can cause potentially life threatening mediastinal complications. CT-scan enables accurate and timely diagnosis and provides valuable indications for treatment. Extraction of esophageal FB

with a rigid endoscope is a good and safe treatment alternative. Non-operative management of esophageal perforation is not an option in the presence of neck and mediastinum abscesses and necessitates a surgical suture and drainage.

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A case of multiple intra-abdominal splenosis with computed tomography and magnetic resonance imaging correlative findings

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Abstract

Hepatic splenosis refers to heterotopic auto-transplantation and implantation of splenic tissue resulting from the spillage of cells from the spleen after splenic trauma or splenectomy. The true incidence of splenosis is unknown, because this entity is usually an incidental finding at surgery. Splenic implants are usually multiple, and can be localized anywhere in the peritoneal cavity. Splenic implants in the peritoneal cavity may be confused with renal tumors, abdominal lymphomas and endometriosis. We describe computed tomography (CT) and magnetic resonance imaging (MRI) findings in a rare case of multiple intra-abdominal splenosis located along the hepatic surface and adjacent to the upper pole of the right kidney, mimicking a renal neoplasm.

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Key words: Abdomen; Computed tomography; Magnetic resonance imaging; Liver; Spleen

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INTRODUCTION

Hepatic splenosis refers to heterotopic auto-transplantation

and implantation of splenic tissue resulting from the spillage of cells from the spleen after splenic trauma or splenectomy, occurring in up to 67% of patients who have a splenic rupture^[1,2]. Splenic implants are usually multiple and can be localized anywhere in the peritoneal cavity, but they usually occur on the serosal surfaces of the small and large bowel, the peritoneum, the mesentery and the diaphragm^[3].

We describe computed tomography (CT) and magnetic resonance imaging (MRI) findings in a rare case of intra-abdominal multiple splenosis located along the hepatic surface and in close proximity to the upper pole of the right kidney, mimicking a renal neoplasm.

CASE REPORT

A 39-year-old man with a history of Crohn's disease was admitted to our institution because of persistent abdominal pain. Physical examination and past medical history were unremarkable except for the previous history of chronic inflammatory bowel disease and an emergency splenectomy performed at the age of 15 after a car accident. An abdominal ultrasound was performed, revealing small bowel wall thickening at the level of the terminal ileum and minimal ascites in the peritoneal cavity. Ultrasound also revealed the presence of a 4 cm solid echogenic mass in the upper pole of the right kidney.

An helical CT examination showed a well-demarcated 3 cm mass in the sub-capsular posterior portion of the seventh segment of the right hepatic lobe adjacent to the upper pole of the right kidney. The mass was hypodense compared with the surrounding liver parenchyma on unenhanced helical CT examination, and showed heterogeneous enhancement in the arterial phase after the administration of contrast material, becoming hypodense compared with the surrounding parenchyma, during the portal (Figure 1) and equilibrium phases. The CT scan also identified two similar nodular lesions medially to the left lobe of the liver, and adjacent to the upper pole of the left kidney and the pancreatic tail (Figure 1).

A contrast-enhanced helical MRI scan was subsequently obtained, which confirmed the presence of a 3 cm hepatic lesion along the posterior surface of the seventh segment and in close proximity to the upper pole of the right kidney. Furthermore, MRI identified two additional lesions along the lateral surface of the right hepatic lobe and more clearly identified the other intra-abdominal nodular implants medially to the left hepatic

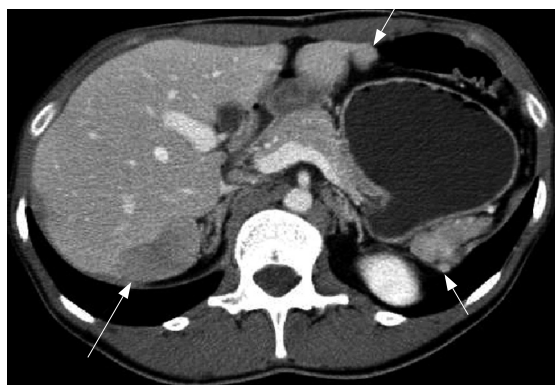


Figure 1 Contrast-enhanced helical CT obtained during the portal phase of acquisition, showing a hypodense 3 cm lesion along the posterior surface of the seventh segment of the right lobe of the liver (long white arrow), and two similar nodular lesions medially to the left lobe of the liver and adjacent to the upper pole of the left kidney and the pancreatic tail (small white arrows).

lobe and adjacent to the upper pole of the left kidney and the pancreatic tail. These lesions appeared hypointense on T1-weighted unenhanced MRI scans (Figure 2A) and slightly hyperintense on T2-weighted images (Figure 2B), showing nonhomogeneous enhancement during the arterial phase, and hypointensity during the portal and equilibrium phases. The patient subsequently underwent surgical resection of the mass described along the surface of the seventh segment of the liver and adjacent to the upper pole of the right kidney, to exclude the presence of a renal neoplasm. Histopathologic examination indeed demonstrated the benign nature of the lesion, which consisted of splenic tissue. In addition, surgical exploration confirmed the presence of additional foci of splenic tissue in the abdominal cavity.

DISCUSSION

Hepatic splenosis or the heterotopic implantation of splenic tissue after splenic trauma or splenectomy is a common but under diagnosed entity, occurring in up to 67% of patients who have suffered from a splenic rupture^[1-4]. The true incidence of this rare condition is unknown, because splenosis is usually an incidental finding at surgery. The implants can be solitary or multiple, and can occur throughout the peritoneal cavity or chest^[3] if splenic rupture is associated with a diaphragmatic tear. The nodules of splenosis do not generally grow to a large size, because they do not have their own blood supply. Although splenic implants are generally asymptomatic, they can lead to recurrent episodes of abdominal pain or small bowel obstruction secondary to adhesive bands of splenic implants. Most patients who undergo surgery for splenosis present to the surgeon with a diagnosis of intestinal obstruction or appendicitis. Although several cases of splenosis have been described in the past^[1,5], this case is the first to describe multiple intra-abdominal heterotopic splenic implants, particularly involving the sub-capsular portion of the liver and the right retroperitoneal space, adjacent to the upper pole of the right kidney, with correlative CT and MRI findings. Differential

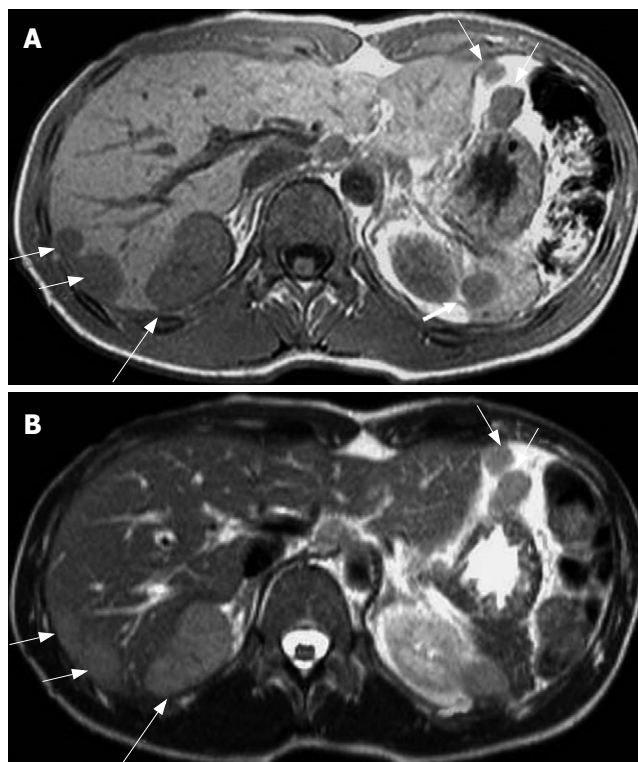


Figure 2 Different image showing a hypointense 3 cm lesion along the posterior surface of the seventh segment of the right lobe of the liver (long white arrow) and 5 additional lesions in the sub-capsular portion of the seventh segment of the liver, medially to the left lobe of the liver and adjacent to the upper pole of the left kidney and the pancreatic tail (small white arrows). A: Unenhanced T1-weighted (TR: 218, TE: 4.6 ms) axial MRI scan; B: T2-weighted (TR: 417, TE: 80 ms) axial image.

diagnoses of splenosis include endometriosis, peritoneal mesothelioma, renal neoplasms^[6], abdominal lymphomas^[7] hepatic adenomas^[8] and peritoneal metastatic implants. Both ^{99m}Tc-red blood cell SPECT scans and ^{99m}Tc-sulphur colloid scans can also be used to differentiate splenosis from malignancies and several reports have described the usefulness of these nuclear medicine techniques to avoid unnecessary surgical intervention^[9,10]. MRI contrast agents composed of super-paramagnetic iron oxide particles that show a tissue-specific bio-distribution to phagocytic reticuloendothelial cells of liver and spleen after intravenous injection, have also been used in the past in patients with splenosis^[3]. These agents produce local inhomogeneities in the magnetic field causing rapid dephasing of transverse magnetization, resulting in a loss of signal intensities on MRI of both ectopic splenic tissue and normal spleen. MRI offers the advantages over nuclear medicine techniques of combining higher spatial resolution with a physiological test of reticuloendothelial cell uptake^[11]. Due to MRI's superior contrast resolution compared with CT, MRI better enabled the identification of multiple intra-abdominal splenic lesions, in particular along the posterior hepatic surface, allowing the presence of a renal neoplasm in the right retroperitoneal space to be excluded. MRI findings coupled with the patient's history of previous splenectomy allowed the correct diagnosis of multiple intra-abdominal splenosis to be made.

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CASE REPORT

Intrauterine midgut volvulus without malrotation: Diagnosis from the 'coffee bean sign'

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Abstract

Fetal midgut volvulus is quite rare, and most cases are associated with abnormalities of intestinal rotation or fixation. We report a case of midgut volvulus without malrotation, associated with a meconium pellet, during the gestation period. This 2.79 kg, 33-wk infant was born *via* a spontaneous vaginal delivery caused by preterm labor. Prenatal ultrasound showed dilated bowel loops with the appearance of a 'coffee bean sign'. This patient had an unusual presentation with a distended abdomen showing skin discoloration. An emergency laparotomy revealed a midgut volvulus and a twisted small bowel, caused by complicated meconium ileus. Such nonspecific prenatal radiological signs and a low index of suspicion of a volvulus during gestation might delay appropriate surgical management and result in ischemic necrosis of the bowel. Preterm labor, specific prenatal sonographic findings (for example, the coffee bean sign) and bluish discoloration of the abdominal wall could suggest intrauterine midgut volvulus requiring prompt surgical intervention.

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Key words: Midgut volvulus; Coffee bean sign; Meconium ileus

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Park JS, Cha SJ, Kim BG, Kim YS, Choi YS, Chang IT, Kim GJ, Lee WS, Kim GH. Intrauterine midgut volvulus without malrotation: Diagnosis from the 'coffee bean sign'. *World J Gastroenterol* 2008; 14(9): 1456-1458 Available from: URL: <http://www.wjgnet.com/1007-9327/14/1456.asp> DOI: <http://dx.doi.org/10.3748/wjg.14.1456>

INTRODUCTION

Midgut volvulus is a condition in which the small bowel or proximal colon twists around the superior mesenteric artery. This condition most commonly presents during the first year of life and has high rates of morbidity and mortality^[1,2]. Midgut volvulus without malrotation is an extremely rare surgical condition, which may also occur during gestation^[3-6]. We recently encountered an unusual case in which intrauterine volvulus occurred with prenatal meconium pellets. Emergent prenatal ultrasonography revealed the presence of the 'coffee bean sign' in this fetus. The patient required resection of a significant amount of necrotic small bowel and treatment with intra-operative saline irrigation. The patient also needed postoperative gastrografin enema due to persistent meconium ileus. Fortunately, the patient survived and has continued to thrive without parenteral nutrition. We discuss the pathogenesis of intrauterine midgut volvulus associated with complicated meconium ileus and the issues surrounding their emergent diagnosis and management.

CASE REPORT

A 27-year-old pregnant woman was referred to our clinic at 33 wk of gestation because of fetal intestinal dilatation found on sonography, and the onset of preterm labor. Routine examinations at a local clinic had revealed a dilated intestine in the fetus, but this had improved spontaneously four weeks earlier. Transabdominal ultrasound on referral showed a segment of markedly dilated fetal intestine, which suggested a closed loop obstruction (Figure 1). Fetal ultrasound measurements were appropriate for the gestational age, and the Doppler indices were normal. No ascites was seen in the fetal abdomen. As it was possible the bowel obstruction would be complicated by intestinal necrosis, preterm delivery was considered beneficial. At 33 wk and two days, a male infant was delivered transvaginally. The patient weighed 2690 g and had Apgar scores of 8 and 9 at 1 and 5 min, respectively.



Figure 1 A fetal sonogram showing dilated bowel loops with the appearance of a 'coffee bean sign'. No ascites was seen in the fetal abdomen.



Figure 2 **A:** Pre-operative infantogram showing a gas shadow only in the stomach, with an absence of any distal gas shadow; **B:** Unenhanced abdominal CT showed meconium (arrow) in the distal small bowel, with mild fluid distension of the proximal small bowel.

The abdomen was distended markedly and there was a bluish skin discoloration on the periumbilical abdominal skin. Nasogastric aspiration recovered 10 mL of bilious material. A rectal examination was normal. Initial laboratory values included a white blood cell count of 26 000/mm³, a platelet count of 416 000/mm³ and prothrombin time of 11 s. Blood gas values from the umbilical artery were pH 7.14, PO₂ 51.3 mmHg and PCO₂ 60.44 mmHg. A plain supine abdominal radiograph did not demonstrate bowel gas, except in the stomach. An emergency computer tomography (CT) scan performed without contrast media revealed marked intestinal dilatation mainly in the left abdomen, and a large amount of hemorrhagic ascites (Figure 2).

Exploration revealed a volvulus of the small bowel with extensive necrosis extending from 40 cm distal to the ligament of Treitz to 15 cm proximal to the ileocecal valve. The distended segment of intestine was twisted at the level of the narrow meconium filled distal ileum (Figure 3). There was no intestinal malrotation, mesenteric defect or atresia. After detorsion of the midgut volvulus, the thick meconium of distal ileum was irrigated by instilling saline with an 8-Fr rubber catheter. The involved loop was then resected and an end-to-oblique anastomosis was constructed between the dilated proximal and smaller

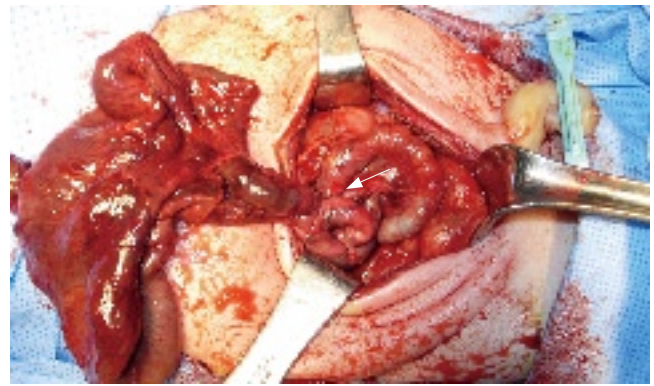


Figure 3 On laparotomy, the infant was found to have a midgut volvulus with necrosis and perforation of the small bowel. The small bowel was found to be twisted at the level of the narrow meconium-filled distal ileum (arrow).

distal bowels by manual anastomosis. On the fourth day after surgery, the patient required treatment with a gastrografin enema to loosen the persisting meconium ileus. A presumptive diagnosis of cystic fibrosis was made postoperatively, based on meconium ileus. There was no family history. To date, the baby has been screened, but all results have been negative. The infant made a remarkable recovery and was able to tolerate enteral feeding with a steady weight gain by four months after surgery.

DISCUSSION

Midgut volvulus is a surgical emergency frequently encountered in neonates. Most cases of volvulus in infants and fetus are associated with intestinal malrotation or congenital anomalies, such as omphalocele, gastroschisis, intestinal atresia or an annular pancreas^[7]. On the other hand, the etiology of volvulus without malrotation is unknown, and associated anomalies are rare^[5]. Several studies have shown that the absence of a segment of small bowel muscle or a mesenteric defect might be associated with this condition^[8-11]. There is no clear explanation for the cause of this event in our patient, because laparotomy did not reveal any intestinal malrotation or congenital mesenteric anomalies. However, we suggest the fetal midgut volvulus and preterm labor was caused by the complicated meconium ileus. Intestinal volvulus might occur when the distended segment of the small bowel becomes twisted at the level of the narrow pellet-filled distal ileum. Gestational volvulus can result in ischemic necrosis, leading to fetal stress, which might activate the release of both adrenal and hypothalamic stress hormones. These might enhance placental, decidual and amniochorionic corticotrophin-releasing hormone release, while premature rupture of fetal membranes and preterm labor can be mediated by placental and membrane prostanoid release^[12,13].

Previous reports have described cases with midgut volvulus in which the fetal sonograms showed intestinal dilatation, a discrete cystic or solid abdominal mass, ascites, peritoneal calcification, polyhydramnios and, typically, the whirlpool or snail sign^[14,15]. Unfortunately, our patient did not show any definitive sonographic sign of midgut

volvulus. Instead, retrospective analysis of the patient's prenatal sonographic imaging revealed a coffee bean sign, which is a specific indicator of sigmoid volvulus in adult patients. Attention should always be paid to the risk of a midgut volvulus with such prenatal sonographic findings.

The nonenhanced CT scan performed after spontaneous vaginal delivery showed excessive hemorrhagic ascites, which was invisible during prenatal sonography. This suggests the bowel necrosis and meconium peritonitis may have developed rapidly during the spontaneous vaginal delivery. Rapid emergency Cesarean section or accelerated delivery should be considered for such expectant mothers who have a history of recurrent closed loop obstruction in the fetus, and who present with acute preterm labor, because the symptoms might occur perinatally with rapid progression to gangrene.

Delays in diagnosis are likely in such cases, as physicians tend to doubt or not suspect the possibility of intrauterine volvulus because it is so rare. Therefore, close prenatal monitoring is necessary if there is any suspicion of typical sonographic signs in the fetus. The adoption of more prompt delivery methods with exploration, avoidance of unnecessary special studies and appropriate postnatal intervention are all essential to reduce the likely morbidity and mortality of intrauterine volvulus associated with complicated meconium ileus.

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Transanal excision of a malignant fibrous histiocytoma of anal canal: A case report and literature review

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Abstract

Malignant fibrous histiocytoma, which is composed of spindle-shaped cells arranged in a pleomorphic and storiform pattern, is rarely found in the colorectum. Although complete surgical excision remains the main stem of therapy, an optimal treatment strategy according to the stage has not been elucidated. We report a case of a 63-year-old woman with an ulcerative lesion in the anorectal junction and a final diagnosis of malignant fibrous histiocytoma. We introduced an access for transanal local excision and adjuvant radiotherapy because the patient refused abdominoperineal resection. No local recurrences or distant metastases were observed 15 mo after the operation. To our knowledge, this is the first case reported in the English literature of a malignant fibrous histiocytoma treated with the transanal local excision and adjuvant radiotherapy. This report showed that this approach is selectively reserved for early-stage malignant fibrous histiocytoma and for those patients who refuse radical surgery because of the risk in a permanent colostomy.

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Key words: Malignant fibrous histiocytoma; Anorectal junction; Transanal local excision

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INTRODUCTION

Malignant fibrous histiocytoma (MFH) is a soft-tissue sarcoma composed of anaplastic-appearing fibroblasts and histiocytes arranged in a storiform collagenous matrix. MFH is the most common type of soft-tissue sarcoma in adults and it usually localizes in the lower extremities, especially the thigh^[1,2]. Colorectal MFH, however, remains extremely rare and only a few cases have been reported worldwide^[3-23]. Surgical resection is thought to be the most effective treatment. Adjuvant chemotherapy or radiotherapy may be advisable, but the optimal histology-specific treatment protocol of this disease has not been clarified.

We report a case of a 63-year-old patient with MFH of the anorectal junction who was treated by transanal local excision and adjuvant radiotherapy. We also briefly discuss the diagnosis, surgical treatment, and adjuvant therapy of this rare case.

CASE REPORT

A 63-year-old woman was referred to our institution because of blood tinged stool and anal mass that was discovered during screening colonoscopy. She had no history of anal surgery or radiation therapy. A well defined mass on the right anterior aspect of the anal canal was palpated *via* digital rectal examination and was located 3 cm from the anal verge. Routine laboratory data and levels of tumor marker, carcinoembryonic antigen, alpha-fetoprotein, and carbohydrate antigen 19-9 revealed no marked abnormalities.

On colonoscopy examination, an ulcerative fungating mass, beginning proximal to the dentate line and extending cranially for about 1 cm, was found (Figure 1). Computed tomography of the abdomen and pelvis did not show the presence of the mass. However, anal magnetic resonance imaging confirmed a tumor of at least 1.5 cm diameter growing into the distal rectal lumen (Figure 2). The surrounding structures appeared to be free from invasion,



Figure 1 On colonoscopy, an ulcerated fungating mass was detected 3 cm from the anal verge.

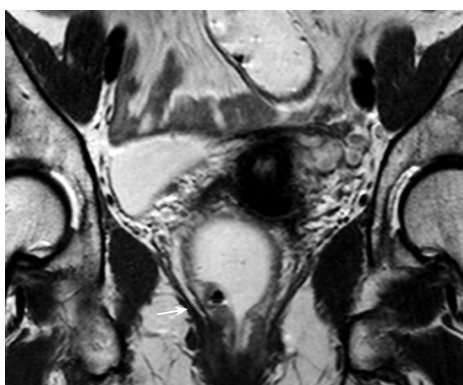


Figure 2 Magnetic resonance imaging shows a T1-weighted of low signal area (arrow) growing in the anterior portion of the anorectal junction.

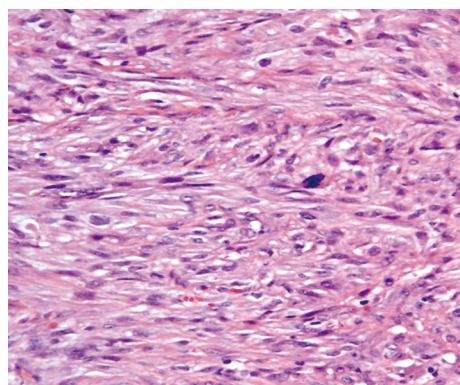


Figure 3 Histological section shows a typical malignant fibrous histiocytoma featuring spindle cells arranged in a storiform pattern (HE, $\times 400$).

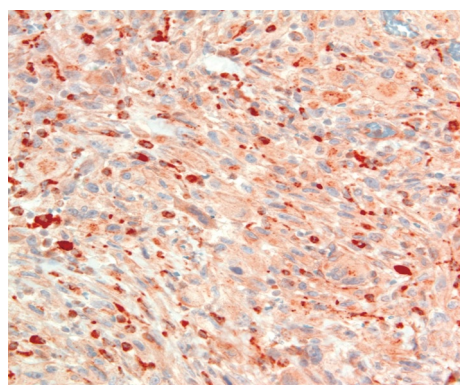


Figure 4 Tumor cells show positive immunostaining for CD68 ($\times 400$).

and we excluded extension into the muscularis propria of the bowel wall. These results were consistent with anal or rectal cancer. However, a colonoscopic biopsy specimen revealed a few giant malignant cells arranged in a sheet like pattern among necrotic granulation tissues. We made a preoperative diagnosis of a malignant spindle-shaped tumor or nonepithelial malignant tumor.

Our first plan, an abdominoperineal resection with end colostomy, was refused by the patient. The alternative, a sphincter-sparing transabdominal approach with colonal anastomosis, seemed to be technically difficult because of the tumor's its low location and possible focal involvement of the dentate line. We decided to use the initial transanal approach to define the tumor character and the risk of aggressive behavior, and to consider a more aggressive treatment if the tumor belonged to a high-risk group. Under general anesthesia, the patient was placed in the jack-knife position, and a transanal local excision was made. The tumor was located in the submucosal layer and was excised completely with just a small amount of adherent fiber of the anal sphincter and a resection-free margin of 1 cm. After excision, we obtained a further resection margin through multiple punch biopsies, which included the proximal and distal margin, right and left lateral margin, and deep margin around the surgical bed. The muscular layer of the surgical bed was closed with Dexon sutures.

Gross pathological examination showed a 1.7 cm \times

1.3 cm \times 0.3 cm fibrous-elastic mass. On the cut section, the tumor was firm in consistency, solid, and yellowish in color. Histological examination showed that the tumor was pleomorphic and composed of atypical spindle cells. These cells were arranged in a storiform pattern (Figure 3). The resection margins of the specimen and all the punch-biopsied tissue were free of tumor. Immunohistochemical stains were positive for CD68, vimentin and negative for cytokeratins, CD117, CD34, alpha smooth muscle actin, desmin, S-100 protein, and HMB-45 (Figure 4). The final histopathological diagnosis was MFH of the anal canal.

The patient had an uneventful postoperative course. No implication in anal continence was observed, and she was discharged on the fifth postoperative day. She received postoperative radiation (60 gray/30 fractions) to the surgical bed. Follow-up studies after 15 mo, including colonoscopy, computed tomography, anal magnetic resonance imaging, and ^{18}F -fluorodeoxyglucose positron emission tomography scan showed no evidence of local recurrence or distant metastasis.

DISCUSSION

MFH was first described in 1963 by Ozello *et al* and was established by O'Brien and Stout in 1964, which refers to a group of soft tissue sarcoma composed of both histiocytic and fibrous elements, often has some common features,

Table 1 Clinical features of malignant fibrous histiocytoma of anorectum reported in literature

Author (year)	Age/sex	Site	Tumor size (cm)	Primary therapy	Adjuvant therapy	Metastasis/recurrence	Outcome (mo. from first op.)	Remark
Verma (1979) ^[3]	38/M	Rectum	12	APR	No	NED	Alive (14)	AV 6 cm
Spagnoli (1984) ^[9]	68/M	Anal cannal	2.0 × 1.6	APR	NA	Lung	Dead (24)	NA
Flood (1989) ^[12]	41/M	Anal cannal	6	APR	Radiation	NED	Alive (16)	Anal sphincter ¹
Singh (1999) ^[21]	55/M	Rectum	4 × 2.5	APR	Chemorad	NED	Alive (46)	AV 4 cm
Present case (2007)	63/F	Anal cannal	1.7 × 1.3	TAE	Radiation	NED	Alive (15)	AV 3 cm

NA: No available information; M: Male; F: Female; APR: Abdominoperineal resection; TAE: Transanal local excision; Chemorad: Adjuvant chemo-radiation therapy; NED: No evidence of disease relapse; AV: Anal verge. ¹Tumor located in the deeper part of anal sphincter (left ischiorectal fossa).

such as pleomorphism and storiform pattern of growth and is accompanied by inflammatory cells^[24,25]. The most frequent site of MFH is the deep tissue of the extremities and trunk. In a retrospective series of 167 patients, 50% of MFH arose in the lower limb, 24% in the upper limbs, 16% in the trunk and 9% retroperitoneum^[26].

The large bowel is an exceedingly rare site of primary MFH. We found only 21 reported cases of primary MFH originating from the colorectum^[3-23]. The median age of these patients, including our patient, is 62 years, with a range of 12-85 years and two pediatric cases have been described^[5,16]. A female predominance has been suggested for extremity MFH, otherwise a review of the literature revealed 19 male and six female patients with colorectal MFH^[20]. Most tumors are large, ranging from 2 cm to 19 cm in diameter (median diameter, 7 cm), and the tumor in our patient is probably one of the smallest tumor observed. The distribution differs from more common types of colorectal cancer^[22]. The tumor locations include nine in the right-sided colon, seven in the transverse and descending colon, three in the sigmoid colon, and four in the anorectum. From the literature, we identified four cases with colorectal MFH which were located in the anorectum (Table 1). All reported lesions were located within 6 cm above the dentate line and were removed by abdominoperineal resection. Two patients treated with radiation as adjuvant treatment survived and were disease free 16 and 46 mo after the initial surgery.

The diagnosis of MFH depends on an accurate differential diagnosis from other sarcomas. The differential diagnosis includes gastrointestinal stromal tumor, fibrosarcoma, leiomyosarcoma, and myxoid sarcoma. Gastrointestinal stromal tumor can be easily identified on the immunohistochemical stains which are positive for CD117 and CD34. The uniform population of spindle cells and the absence of histiocytoid cells and storiform pattern areas may aid the diagnosis of fibrosarcoma. The presence of cells with perinuclear vacuoles, intracytoplasmic glycogen, and desmin positivity is suggestive of leiomyosarcoma; and finally, a diffuse, prominent myxoid background with interspersed atypical pleomorphic spindle cells is reminiscent of myxoid sarcoma. It was reported that MFH frequently expresses vimentin, actin, alpha 1-antitrypsin and CD68^[27]. Therefore, MFH in the current patient was diagnosed based on the histological findings of the characteristic storiform pattern and the positive immunohistochemical staining for vimentin and CD68.

The efficacy of radiation therapy is well established in

the treatment of soft tissue sarcoma of extremities^[28]. After postoperative radiation therapy, the local recurrence rate is lower (27%) than in patients with similar tumor-related characteristics who were not treated with radiation (58%). The effect of adjuvant radiotherapy to gastrointestinal MFH is unclear. In the current case, there were concerns that a microscopic residual tumor infiltrating through the circumference tissue might remain because we could not perform the radical resection. Moreover, as local recurrence is found in the most common pattern of colorectal MFH, we decided to perform adjuvant radiation therapy in our patient.

Previous studies reported that radical excision, like amputation, decreases the local recurrence rates in soft tissue sarcoma of the extremities. However, limb salvage with more conservative surgery or function sparing-surgery is performed increasingly. As for anorectal gastrointestinal stromal tumors, some authors suggested that most tumors with a diameter less than 5 cm treated with local excision and radical surgery including abdominoperineal resection are associated with high mortality and morbidity^[29]. It is thought that the natural history of these patients partly excludes the benefit of radical surgery. In our patient, because the primary lesion was detected at an early stage by a screening colonoscopy, we could excise the tumor completely using the transanal approach. Fortunately, no clinical signs of local recurrence or metastasis were detected at the follow-up examination after 15 mo. Based on our experience, we think this approach enables an alternative for early stage patients who cannot tolerate radical surgery or refuse the abdominal-perineal resection.

Due to the rarity of anorectal MFH, there is an absence of histology-specific treatment protocol at present. More data are needed about the oncologic outcomes of sphincter preserving operations with multimodality therapy, as well as the feasibility and safety of salvage operations in case of local recurrence.

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Overlap of reflux and eosinophilic esophagitis in two patients requiring different therapies: A review of the literature

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Abstract

Eosinophilic esophagitis (EE) and gastroesophageal reflux disease (GERD) have overlapping clinical, manometric, endoscopic and histopathologic features. The diagnosis of EE is nowadays based upon the presence of 15 or more eosinophils per high power field (eo/HPF) in esophageal biopsies. We report the cases of two young males suffering from dysphagia and recurrent food impaction with reflux esophagitis and more than 20 eo/HPF in upper-mid esophagus biopsies, both of which became asymptomatic on proton pump inhibitor (PPI) therapy. The first patient also achieved a histologic response, while EE remained in the other patient after effective PPI treatment, as shown by 24-h esophageal pH monitoring. Topical steroid therapy combined with PPI led to complete remission in this latter patient. GERD and EE may be undistinguishable, even by histology, so diagnosis of EE should only be established after a careful correlation of clinical, endoscopic and pathologic data obtained under vigorous acid suppression. These diagnostic difficulties are maximal when both diseases overlap. Limited data are available about this topic, and the interaction between EE and GERD is a matter of debate. In this setting, upper-mid esophagus step biopsies and esophageal pH monitoring of patients on PPI therapy are pivotal to evaluate the role of each disease. A PPI trial is mandatory in patients with a histopathologic diagnosis of EE; in those unresponsive to PPI treatment, EE should be suggested. However, a clinical response to PPI may not rule out quiescent EE, as shown in this report.

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Key words: Eosinophils; Eosinophilic esophagitis;

INTRODUCTION

Eosinophilic esophagitis (EE) is an emerging under-diagnosed disease in adults, associated with allergic or asthmatic disorders, which typically affect males in the second to fourth decades of life. The main symptoms are dysphagia, heartburn and chest pain, and it may be present in half of all adults with bolus food obstruction of the esophagus^[1,2]. The classical endoscopic appearance of the esophagus is polymorphic and includes papular elevations, whitish exudates, corrugation, longitudinal furrows, undulated mucosa, reddening, multiple contraction rings and strictures, but may also be normal in 25% of cases^[1,3]. Esophageal manometry may show non-specific motor disturbances, such as hypoperistaltic and hyperperistaltic disorders, or nontransmitted or simultaneous contraction waves^[3].

Thus, EE and gastroesophageal reflux disease (GERD) can not be distinguished based on clinical, manometric or endoscopic features. Mild eosinophilic infiltration of the distal esophagus, usually less than 7-10 eosinophils per high power field (eo/HPF), is a hallmark of GERD, so on histological confirmation, which is required for the diagnosis of EE, dense eosinophil infiltrates (> 15-20 eo/HPF) must be observed along the length of the esophagus, in the absence of eosinophilic gastroenteritis, parasites or fungal infections, vasculitis or granulomatous diseases. The interaction between EE and GERD seems complex, and recently the importance of GERD in EE has been suggested due to its higher than expected prevalence, and the report of the resolution of EE with proton pump inhibitor (PPI) therapy^[4,5].

CASE REPORT

A 21-year-old man (case 1) and a 35-year-old man (case 2) with no prior medical history both presented with acute-onset dysphagia and a complete inability to swallow saliva after food impaction in the mid and proximal esophagus, respectively. Both patients related a 2-year history of non-progressive intermittent dysphagia to solids and recurrent food impaction, which improved after drinking water. They had no previous asthma, allergic disorders or seasonal variations in their symptoms. Neither complained of heartburn, chest pain, or regurgitation. No peripheral eosinophilia or elevation in IgE level was detected in blood samples, while gastric and duodenal biopsies were normal.

Case 1

A meat bolus impacted at 32 cm from the incisors was removed in the emergency room with a polypectomy snare by endoscopy, which revealed no esophageal mucosa abnormalities (Figure 1A), but no biopsies were taken. A previous upper endoscopy due to meat impaction and a barium swallow revealed the esophagus to be normal. Esophageal manometry demonstrated severe dysmotility in the distal esophagus, with two thirds of all esophageal contractions waves measured being simultaneous and interrupted (Figure 1B) and a normal lower esophageal sphincter pressure. The patient refused 24 h esophageal pH monitoring because of severe intolerance. On this basis, a third esophagoscopy was performed to rule out EE. Upper endoscopy showed three mucosal breaks in the distal esophagus, more than 5 mm long, but not continuous between the tops of adjacent folds, compatible with grade B (Los Angeles classification) reflux esophagitis, while the rest of the mucosa was normal. Upper-mid esophagus step biopsies showed prominent eosinophil micro-abscesses (Figure 1C) and a high density eosinophilic infiltrate (31 eo/HPF, magnification $\times 400$), with a predominantly superficial distribution (Figure 1C, box), mid basal zone hyperplasia and intercellular edema, which also contributed to the suspected diagnosis of EE. A 2-mo course on PPIs (omeprazole 40 mg/d) without steroids led to clinical, manometric and pathologic remission (Figure 1D).

Case 2

Emergency endoscopy showed a mixed vegetable bolus impacted at 23 cm from the dental margin, which was removed with a polypectomy snare and a Roth retrieval net. A normal caliber upper-mid esophagus with undulated mucosa was detected (Figure 2A), as well as a peptic fibrotic stricture at the cardias (Figure 2B), which was dilated using gentle pressure of the endoscope, with a medium size sliding hiatal hernia below. Esophageal step biopsies from the upper-mid esophagus showed severe lamina propria fibrosis (Figure 2C) and a dense intraepithelial eosinophilic infiltration (37 eo/HPF, magnification $\times 400$) predominantly in the luminal surface of epithelium, with intercellular edema, basal cell hyperplasia and papillae elongation (Figure 2C, box). After a 2 mo course of PPIs (omeprazole 40 mg per day) the patient became asymptomatic, but endoscopic and all pathologic features remained at follow-up endoscopy (Figure 2D), despite PPI therapy, including 32 eo/HPF

with degranulating superficial eosinophils. 24-h esophageal pH monitoring of the patient on PPIs ruled out persistent acid reflux (total time pH < 4.0 , 4%, DeMeester score 3) (Figure 2D, box). Due to these findings, a 3 mo course of fluticasone propionate, at a dosage of 500 μg per 12 h, on top of PPIs, was started. The patient finally achieved histopathologic remission with this combined therapy.

DISCUSSION

Until recently, diagnosis of EE has been based upon the presence of more than 15-20 eo/HPF in esophageal biopsies. Due to the patchy distribution of EE, multiple-step biopsies of the mid and proximal esophagus are advisable to maximize diagnosis yield, since GERD may also induce esophageal eosinophilia, normally less than 7-10 eo/HPF, although this has been reported to be typically confined to the distal esophagus^[1,3]. The diagnosis criteria of EE (> 15 -20 eo/HPF) are based on the unlikelihood to observe an eosinophil count above this cut-off value in individuals with GERD and other secondary causes of eosinophilia. This cut-off value is arbitrary, and strict consensus on histopathologic diagnostic criteria of EE is lacking as a recent systematic review has shown a wide variety of eosinophil count cut-off points, methods for counting eosinophils and esophageal biopsy protocols in the literature on EE^[6,7]. In this setting, eosinophil counts may lose diagnostic power, so the prominence of eosinophils towards the luminal surface, eosinophil micro-abscesses (defined as a cluster of 4 or more eosinophils), degranulating eosinophils and lamina propria fibrosis have been reported as secondary pathologic markers of EE^[7,8]. Moreover, and to complicate the scenario, there has been a recent report of three cases fulfilling all clinical, endoscopic and histopathologic diagnosis criteria of EE, who achieved complete response to PPI therapy^[5]. To avoid these diagnostic difficulties, consensus recommendations have recently been made by the First International Gastrointestinal Eosinophil Research Symposium (FIGERS) subcommittees^[9], which define EE with three statements: (1) Symptoms including, but not restricted to food impaction and dysphagia in adults, and feeding intolerance and GERD symptoms in children; (2) 15 or more eosinophils/HPF; (3) exclusion of other disorders associated with similar clinical, histological or endoscopic features, especially GERD, with PPI treatment or esophageal pH monitoring.

The report by Ngo^[5] and case 1 of this manuscript highlight that, in contrast to previously reported data^[1,3], it is possible that GERD may cause identical high-density esophageal eosinophilia and histopathologic changes suggestive of EE in both the proximal and distal esophagus. According to initial endoscopic and pathologic data, case 1 would have been unequivocally diagnosed of EE. However, concomitant esophageal reflux lesions at the third endoscopy and complete remission on PPI treatment suggest the main diagnosis was GERD, which may have been triggering EE, or simply GERD mimicking EE histologically. Conversely, case 2 also had histological features of EE and esophageal acid reflux lesions, and became asymptomatic on PPI therapy, which made

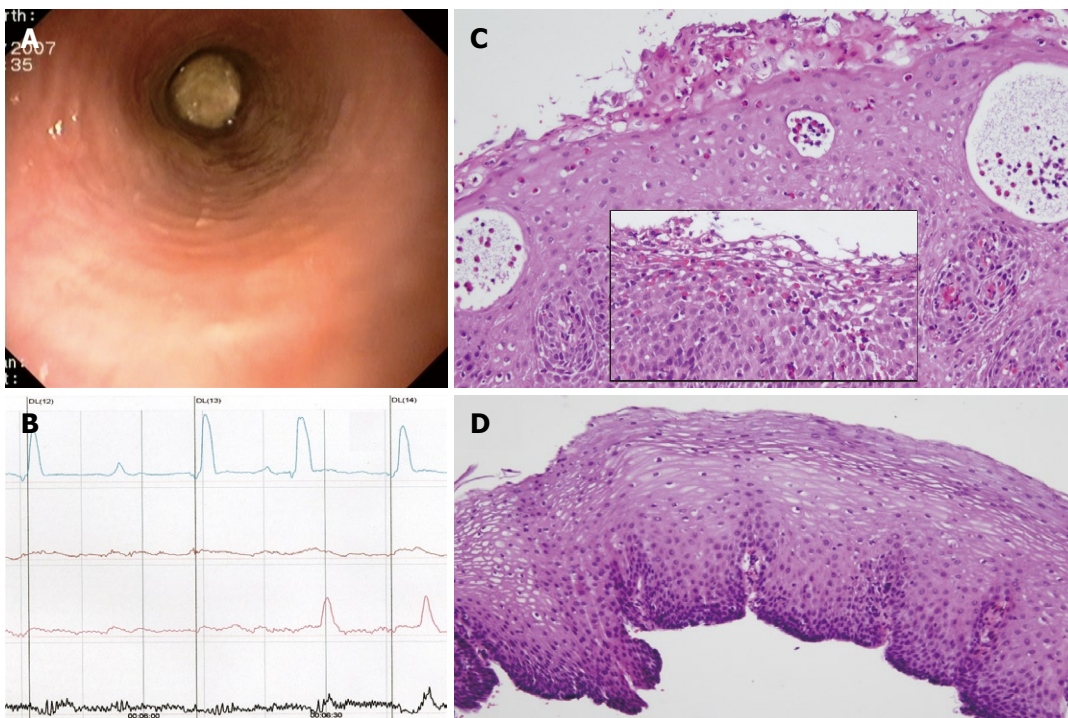


Figure 1 Case 1. **A:** Emergency endoscopy showing a meat bolus impacted at mid-distal esophagus with normal mucosa; **B:** Esophageal manometry demonstrated that two thirds of all contractions in the distal esophagus were simultaneous or interrupted contraction waves; **C:** Prominent eosinophil microabscesses in upper-mid esophagus biopsies (HE, $\times 100$). In the box and on top of the image, a dense eosinophilic infiltrate (31eo/HPF $\times 400$) predominantly spread over the superficial layers can be observed (HE, $\times 200$); **D:** Normal upper-mid squamous epithelium after PPI therapy (HE, $\times 100$).

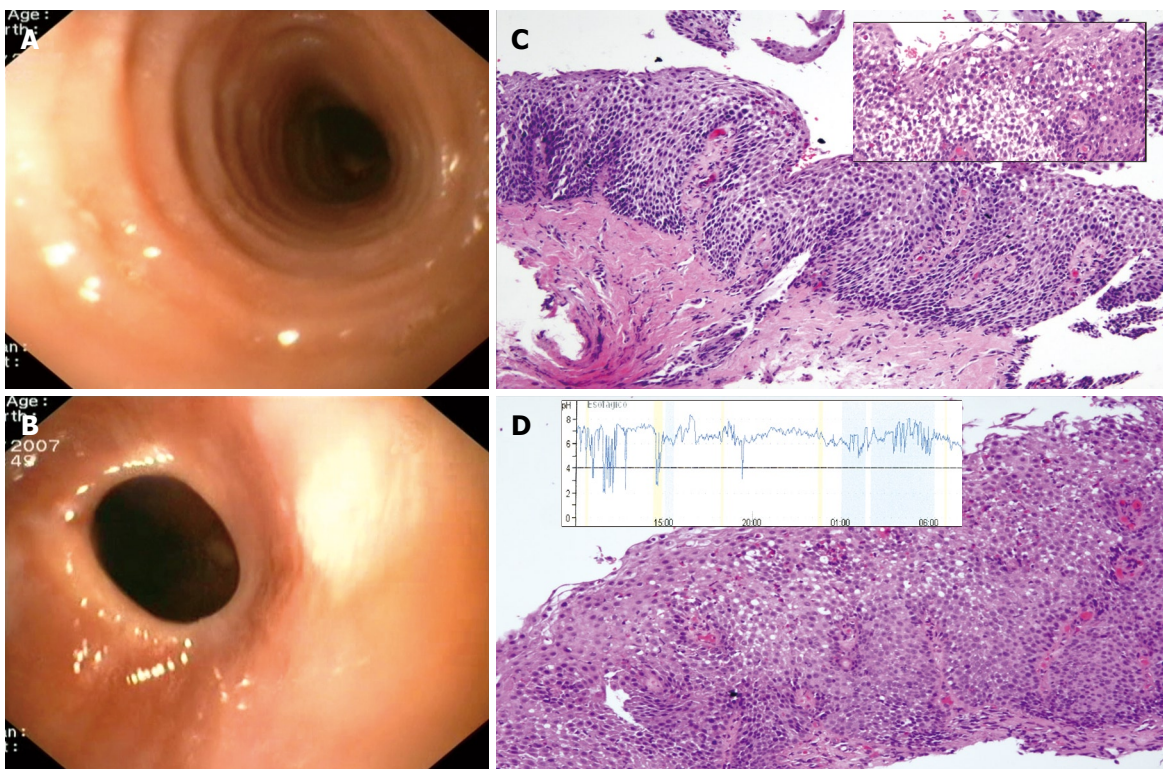


Figure 2 Case 2. **A:** Endoscopic view of the upper esophagus showing multiple concentric rings resembling mucosal undulations; **B:** Endoscopic picture of a peptic stricture at cardias 2 mo after dilation, with gentle pressure of the endoscope; **C:** Biopsies of a multiringed esophagus demonstrating severe lamina propria fibrosis at the bottom of the image, as well as marked papillae elongation and basal zone hyperplasia (HE, $\times 100$). In the upper right box, intense intercellular edema, basal zone hyperplasia and dense eosinophilic infiltrate (37eo/HPF, $\times 400$) towards the surface strata (HE, $\times 200$); **D:** Persistent histopathologic features (32 eo/HPF, $\times 400$) in spite of effective PPI therapy, as shown by 24 h pH esophageal monitoring (box).

GERD the most probable diagnosis. However, persistent histopathologic features on effective PPI therapy and resolution on topical steroids showed the simultaneous existence EE and GERD.

These cases emphasize the importance of thoughtful consideration of clinical and histopathologic responses to acid blockade on the whole, and not only eosinophils count, to establish an accurate diagnosis of EE^[10,11]. The relationship between EE and GERD in adults is a controversial topic nowadays. Some authors defend their unrelated co-existence while others try to search for a plausible casual association. Contradictory data reported on the frequency of acid reflux observed by 24 h esophageal pH monitoring described in adults with EE (38%^[4] against 11%^[8]) does not clarify the situation. It has been suggested motor esophageal changes related to EE may contribute to GERD by decreasing lower esophageal sphincter pressure and delaying esophageal clearance, which would expose longer esophageal mucosa to the acid noxa^[10,11]; this theory may explain the symptomatic response in case 2 under PPI therapy, although endoscopic and pathologic features remained. On the other hand, the previously mentioned report by Ngo^[5] and case 1 of the present manuscript, with documented complete responses of EE to PPI therapy, support the hypothesis that, at least in a small subset of patients with EE, GERD may cause EE, probably by means of an acid-mediated increase in epithelial permeability, allowing immune cell recruitment or access to allergenic peptides^[10,11]; in this setting, PPI could represent an effective therapy for both diseases. In patients with confirmed EE, alimentary allergic study is warranted since an elimination diet, mainly based on skin-prick tests, has demonstrated its effectiveness in terms of clinical and pathologic remission^[12]. In case 2, there were no food allergens detected in a subcutaneous allergic test.

The real interplay between these two diseases is still to be elucidated. Nevertheless, when EE and GERD overlap, the clinical, endoscopic and histologic findings are non-specific in distinguishing both entities. Despite the growing recognition of EE in recent years, the more alert we are to EE, maybe the more we are likely to miss GERD. Indeed, the estimated prevalence of probable EE is 1% in the general population^[13], while GERD is a much more frequent condition which may affect 10%-20% of adults in Western countries^[14]; on this basis, the likelihood to find unrelated GERD in patients with EE is high, especially if we consider that they both commonly affect young males. Then, irrespective of diagnosis criteria of EE and the validation of the relationship between GERD and EE, it is mandatory an initial trial of PPI therapy^[9-11] for patients with histopathologic diagnosis of EE. In those unresponsive to acid suppression, EE should be suggested. However, a clinical response to PPI may not either preclude underlying EE, as demonstrated in case 2.

In conclusion, we report two cases in which reflux esophagitis and EE overlap, which were similar in terms of symptoms, endoscopic reflux lesions, motor esophageal alterations, high-density eosinophilia in upper-mid esophagus biopsies and good clinical response to PPI, but with different endoscopic and histopathologic outcomes after PPI therapy. This fact may reflect that

the interaction between these diseases may be more complex than originally thought and may depend more on individual patient characteristics. An initial trial of PPI therapy in patients with clinical, endoscopic and pathologic findings of EE is warranted. Lack of a response to PPI may reinforce a diagnosis of EE, but a clinical response to PPI may not rule out quiescent EE, as shown in this report. Esophageal pH measurements and histopathologic data on patients on PPI treatment are pivotal in cases with overlapping GERD and EE in order to evaluate the role of each disease. Due to the fact that EE is an uncommon disease, multicentric studies are needed to establish the real incidence and prevalence of EE and GERD in EE, and to clarify the effects of GERD, food allergy and EE on esophageal eosinophilia.

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Complications of extrahepatic echinococcosis: Fistulization of an adrenal hydatid cyst into the intestine

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Abstract

Echinococcal cysts are usually found in liver and lungs, but any other organ can potentially be involved. Extrahepatic disease due to hydatid cyst may develop in the abdominal and pelvic cavity, aside from in other less common locations, which may make both diagnosis and treatment more complex. We present a rare case of extrahepatic echinococcosis in a 70-year old patient with a 4-d history of dull abdominal pain, anemia within the transfusion range and fever. She underwent surgery for left renal hydatid cysts 30 years ago. After non operative treatment, imaging studies showed a calcified hydatid cyst in a retrogastric location communicating with a proximal jejunal loop. En-block resection of the mass together with the adrenal gland was performed including closure of the enteric fistula. Anatomic pathology confirmed the diagnosis of a calcified hydatid cyst of left adrenal origin. Surgery is the treatment of choice and most authors recommend removal of cyst and adrenal gland.

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Key words: Echinococcosis; Adrenal cyst; Surgery

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Ruiz-Rabelo JF, Gomez-Alvarez M, Sanchez-Rodriguez J, Rufian S. Complications of extrahepatic echinococcosis: Fistulization of an adrenal hydatid cyst into the intestine. *World J Gastroenterol* 2008; 14(9): 1467-1469 Available from: URL: <http://www.wjg-net.com/1007-9327/14/1467.asp> DOI: <http://dx.doi.org/10.3748/wjg.14.1467>

INTRODUCTION

Although hydatid cysts can occur in any location, echinococcosis is usually found in the liver and lung. Extrahepatic hydatidosis has been described in the peritoneal cavity, retroperitoneum, spleen, kidney, adrenal glands and even in the spine, myocardium and abdominal wall^[1,2]. Most abdominal extrahepatic hydatidosis patients usually present with a clinical picture of abdominal pain or discomfort, or with more specific symptoms, such as an anaphylactic reaction or fever. Diagnostic difficulties are evident in these cases, especially when the disease evolution is unknown and neither clinical nor analytical examinations indicate a hepatobiliary source of the symptoms^[3]. Complications resulting from this disease are uncommon. Patients with intestinal obstruction have been described as being exceptional cases of extrahepatic hydatidosis. Surgery is considered the treatment of choice by most authors^[4].

We present here a case of fistulization of an adrenal hydatid cyst into an intestinal loop and review the presentation, diagnosis and surgical treatment of extrahepatic echinococcosis in such a location.

CASE REPORT

A 70-year old patient with a history of heart failure and chronic atrial fibrillation visited our hospital for treatment. She underwent surgery for left renal hydatid cysts 30 years ago. Physical examination revealed that she had an incised hernia and history of surgery for gastric ulcer, appendectomy and left hip fracture.

The patient arrived at the Emergency Department with a 4-d history of dull abdominal pain, more intense in the left hypochondrium, and a temperature of 38°C. On examination, a mass and pain in the left epigastrium-hypochondrium were observed on palpation. Blood tests revealed leukocytosis (15 000) with discrete neutrophilia and eosinophils within normal values as well as anemia within the transfusion range (Hb: 7.3; HT: 22). The urinalysis was normal. A simple abdominal X-ray showed a calcified mass in the left epigastric region without signs of intestinal perforation or obstruction. While she was on observation in the hospital, the patient was hemodynamically stable at all times after the transfusion of two units of packed red blood cells. Given the medical history and clinical picture of the patient, an abdominal CT scan was performed, revealing a 9-cm mass with peripheral calcification, wall thickening and central partial fluid image, which seemed to be related to the left kidney's

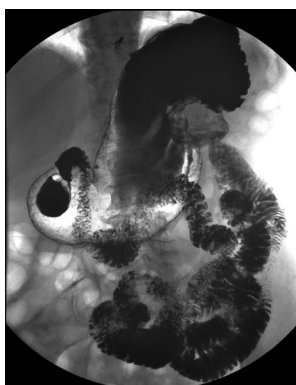


Figure 1 Gastroduodenal transit study showing a calcified mass in a retrogastric location communicating with a proximal jejunal loop.

upper pole, in contact with the stomach which was impressed on its posterior wall. An inflamed-looking loop was observed in the left hypochondrium, with calcification inside and adjacent fluid, which showed no cleavage plane and was highly suspected of being a fistula. Serum IgE (ELISA) was tested for echinococcus and later read as being within the normal range. The patient's evolution during hospitalization was satisfactory. Her fever subsided after antibiotic treatment and strict diet. Subsequent blood tests were normal and no further transfusion was required. In an outpatient setting, a gastroduodenal transit study was performed, showing a calcified hydatid cyst in a retrogastric location communicating with a proximal jejunal loop. No intraluminal repletion defects were observed in the remaining loops (Figure 1). Following diagnosis of a left adrenal primary cyst with a possible hydatid origin, complicated by a fistula of jejunal loop, the patient was surgically intervened *via* a medium laparotomy with an abdominal approach, which allowed for the confirmation of a 12 cm × 9 cm calcified cyst in the left kidney's upper pole, with fistulization into a proximal jejunal loop through the inflamed supramesocolic tract. En-block resection of the mass together with the adrenal gland was performed, avoiding opening of the said mass at all times, including closure of the enteric fistula (Figure 2). No perioperative anti-helminth treatment was needed because there was no surgical dissemination, and no peritoneal involvement of the rest abdominal cavity was observed during surgery. The patient was discharged 12 d after surgery, with a favorable outcome. Anatomic pathology confirmed the diagnosis of a calcified hydatid cyst of left adrenal origin.

DISCUSSION

Sixty to seventy percent of hydatid cysts involve the liver, 5%-15% involve the lung and only 0.5% involve the adrenal glands, an uncommon location for a primary cyst not related to a generalized hydatidosis^[5].

Diagnostic difficulties arise when the evolution of the disease appearing outside the abdominal cavity (myocardium, spine, thyroid gland) is unknown. Abdominal cysts usually have a non-specific clinical presentation and their most common symptom is abdominal pain^[6]. However, other symptoms may be experienced, such as pain in the renal fossa, relatively non-specific gastrointestinal symptoms (dyspepsia, nausea, vomiting, constipation) or even a mass palpable on physical examination^[7]. Sudden

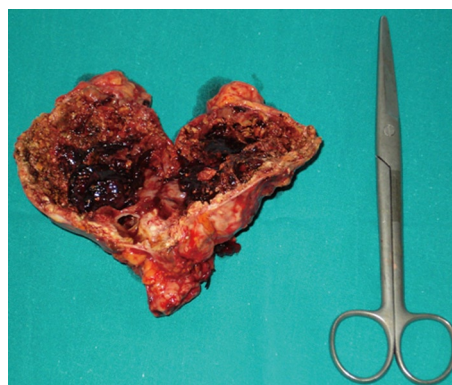


Figure 2 Surgical specimen showing en-block resection of the hydatid cyst together with the adrenal gland.

abdominal pain in such patients may be an indicator of intracystic hemorrhage, rupture or infection, and intracystic hemorrhage is the potentially most dangerous complication^[8,9]. The diagnosis of hydatid cyst in our patient was easy due to her history of hydatidosis and a palpable mass felt in the epigastrium on physical examination. Simple X-ray, necessary in any urgent admission due to fever and abdominal pain, revealed a calcified cyst in the said location which, together with the fever and leukocytosis, indicated a super-infection of the same location. As in the case in question, Akcay^[10], described 9 patients with adrenal hydatid cysts. All plain abdominal X-rays revealed calcifications between the 12th thoracic and the 1st lumbar vertebrae, which is probably related to the silent growth and long evolution of this type of cyst over time. Abdominal CT scan and ultrasound usually confirm the diagnosis with 93%-98% sensitivity for ultrasound and 97% for CT^[11]. It was reported that the presence of calcifications in the adrenal mass greatly supports a diagnosis of hydatid cyst in this location^[11,12]. In our case, the study with barium contrast was important, since the patient presented with recurrent fever episodes and elimination *via* the feces of echinococcus hydatids. We found this study very useful both to confirm the enteric fistula and to indicate surgery. Even though this is an uncommon complication, it might be interesting to bear these diagnostic tests in mind, particularly in patients with a history of extrahepatic hydatidosis surgery with suspected disease recurrence and a clinical picture of abdominal cyst complicated by abdominal obstruction and super-infection. New laboratory tests for echinococcus such as HAI, ELISA or IgE assist in diagnosis, but their negative results, described in these cases by some authors^[5,11] do not rule out the presence of disease. It is mandatory to systematically use imaging studies (CT, ultrasound). It must be borne in mind that aside from imaging and laboratory methods, diagnosis is confirmed by macroscopic and microscopic examination of the surgical specimens, which will show the germinal membrane with the daughter hydatids and scolices of cysticercus in intracystic fluid.

Just as in hepatic and pulmonary hydatidosis, surgery is the treatment of choice^[13]. Most authors recommend removal of cyst and adrenal gland as the safest and most reliable option^[5,14]. This is normally the case because cysts

are usually large, and there is total or partial destruction of the gland on which it lies, and even, as in our case, destruction of surrounding structures and organs. Different incisions have been employed as approaches to the adrenal gland. Most authors do not recommend a laparoscopic approach in case of complex cysts^[15]. In order to prevent surgical dissemination, the procedure should be carefully carried out, providing adequate protection of the surgical field and instilling hypertonic saline solution prior to cyst removal. Albendazole (10 mg/kg) or mebendazole (50 mg/kg) should be administered for a month following surgery in case of suspected peritoneal dissemination or when a peritoneal cyst is removed during surgery^[16,17].

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S- Editor Zhu WL L- Editor Wang XL E- Editor Wang HF



ANNOUNCEMENT

The article published in *WJG* 2005; 11(7): 931-937 partly duplicated an article previously published in *Critical Reviews in Eukaryotic Gene Expression* (2004; 14: 183-202)

From the editor

On November 7, 2007, we received an E-mail from He Zhi-Gang (hezhi-gang@gmail.com) reporting a case of duplicate publication. After our evaluation we are able to confirm this claim. The details are noted below.

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The article published in *WJG* 2005; 11(7): 931-937 partly duplicated an article previously published in *Critical Reviews in Eukaryotic Gene Expression* (2004; 14: 183-202). *World J Gastroenterol* 2008; 14(9): 1470-1471 Available from: URL: <http://www.wjgnet.com/1007-9327/14/1470.asp>

E-MAIL CONTENT OF CLAIMANT (TRANSLATED)

Respected editors

The article published in *World Journal of Gastroenterology* (*WJG*) Volume 11 Issue 7 (pages 931 to 937) by Ting-Ting Li *et al* in 2005 plagiarized an article appearing in *Critical Reviews in Eukaryotic Gene Expression* (*CREGE*) (Volume 14 Issue 3, pages 183 to 202, 2004). The attached two files are copies of both articles, with the plagiarized part highlighted in yellow. Zhi-Gang He.

DETAILS OF DUPLICATE PUBLICATION

Different patterns of duplicate publication

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Li TT, Zhao LN, Liu ZG, Han Y, Fan DM. Regulation of apoptosis by the papillomavirus E6 oncogene. *World J Gastroenterol*. 2005 Feb 21; 11(7): 931-937. Review. PMID: 15742391 [PubMed-indexed for MEDLINE]
<http://www.wjgnet.com/1007-9327/11/931.asp>

An already published article

Xueli Fan, Jason J Chen, Department of Medicine, University of Massachusetts Medical School, 364 Plantation Street, Worcester, MA 01605-2324, United States. jason.chen@umassmed.edu

Fan X, Chen JJ. Regulation of cell cycle progression and apoptosis by the papillomavirus E6 oncogene. *Crit Rev Eukaryot Gene Expr*. 2004; 14(3): 183-202. Review. PMID: 15248815 [PubMed-indexed for MEDLINE]

ANALYSIS OF DUPLICATED MANUSCRIPT

Both manuscripts are reviews on the molecular basis for E6-induced cell proliferation and apoptosis, and most parts of the article published by *WJG* are exact copies of the manuscript published by *CREGE*, without acknowledgment, and including the same sentences, words and even punctuation marks. The yellow highlighted parts in Figure 1 show the highly similar parts in the article published by *WJG*.

Based on this analysis, *WJG* considers this a typical case of duplicate publication, and is prepared to take strict, appropriate action against such behavior.

LETTER FROM THE AUTHOR

Dear Editor,

Here I appreciate you can give me an opportunity to explain this.

I once worked in Jason Chen's lab. This review was invited by *Critical Reviews in Eukaryotic Gene Expression* (*CREGE*). At that time, he asked me to write the apoptosis part, and promised me a first author. Therefore I compiled this review and the manuscript was submitted. After that I didn't get any information on whether or not it has been accepted or not. After I came back from US, there is some unhappy personal issues happened between Jason Chen and me. He threatened to remove my name in the manuscript, but because considering this review is a personal intellectual product, not experiment related, I told him that I had the right to publish it myself. After that the revised paper was submitted to *WJG* and published.

Please refer to the following emails from Editor of *CREGE* for details about the manuscript. There are two points which can be concluded from the following information: The first, I was enlisted as the first author even at TOC of the print copy; the second, Jason Chen asked the editor to remove my name without my permission.

I consider the fact is that Jason Chen neglected my



Figure 1 Snapshot of manuscript (7 pages total) published by WJG. The yellow parts were copied directly from the manuscript published by CREGE.

contribution and published my intellectual product without my permission.

Please contact Editor of CREGE for confirmation. If you do want to publish the attached pdf announcements, I would appreciate you can add the information supplied in this email.

I will be willing to be of any further assistance.

Zhiguo Liu
2008-02-01

Letter from Zhi-Guo Liu to Vicky Lipowski

Dear Matthew Friedman or the person in charge,
On the journal of Critical Reviews in Eukaryotic Gene Expression published in 2004 volume 14(3), there is a paper published entitled Regulation of cell cycle progression and apoptosis by the papillomavirus E6 oncogene (page 183-202). I was the first author during the submission. But I found my name is not in the author list of the final paper. Would you like to check it out for me what happened? Is it a mistake or something else? I would really appreciate your help. Thanks. If you are not the person in charge of this issue, can you forward this email to the person I should talk to?

Have a nice day.

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Reply 1

Dear Dr Liu,
I am checking the error that occurred in CRE 1403. I see that

you are listed in the TOC of the print copy but not with the article. In the meantime I will have the files corrected and update the website as soon as possible. I sincerely apologize for this error and thank you for bringing this to my attention.

Vicky Lipowski
Production Manager

Reply 2

Dear Prof Liu,
Upon looking further into your request concerning the article "Regulation of cell cycle progression and apoptosis by the papillomavirus E6 oncogene (CRE 1403-page 183-202)", I have been informed that the corresponding author felt it was appropriate to move your name from the author list to the acknowledgement section. If you wish to pursue this further you will need to contact Professor Chen. Unfortunately, I cannot correct or issue an erratum without the consent of the corresponding author.

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Letter from Jason J Chen to WJG editor

Dear Editor,
I appreciate the courage of you and He Zhi-Gang to bring up the issue, although my self has long been decided not to pursue it further because I think Zhiguo Liu is a talented physician scientist and should have the opportunity to continue his research. Zhiguo Liu was indeed listed as the first author at beginning. His name was included as a bonus mainly for his work in the laboratory, although his contribution to the manuscript may not be sufficient as an author. Once he was no longer deserved the first authorship, his name was moved to the third position. After he refused to accept the arrangement, his name was moved from the author list to acknowledgement. He was notified about these arrangements.

The only contribution Zhiguo Liu made to the review article was preparation of figures under my guidance. BTW, we did not use his figures for the final version of the article. He did not write the apoptosis part or anything else for the review article. Careful readers should be able to compare the article with my previous review article [Biochimica et Biophysica Acta 1378 (1998) F1-F19] as well as the non-duplicated part of the WJG article for consistency of style.

I noticed that you have changed the word "plagiarize" in the previous version of the announcement to "duplicate" in the current announcement. This is fine with me. It is my fault to have listed him as the first author at the first place and it is awkward to switch the authorship afterwards. I believe we all learnt a lesson from this incident. It is my hope that we leave this behind, let the readers make their own judgments, and move forward.

Jason J Chen
2008-03-01

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Many reviewers have contributed their expertise and time to the peer review, a critical process to ensure the quality of *World Journal of Gastroenterology*. The editors and authors of the articles submitted to the journal are grateful to the following reviewers for evaluating the articles (including those published in this issue and those rejected for this issue) during the last editing time period.

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Meetings

Events Calendar 2008-2009

FALK SYMPOSIA 2008

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

International Gastroenterological Congresses 2008

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

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

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

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

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

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

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

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

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

April 23-27, Milan, Italy
 43rd Annual Meeting of the European

Association for the Study of the Liver
www.easl.ch

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

May 18-21, San Diego, California, USA
 Digestive Disease Week 2008
 June 4-7, Helsinki, Finland

The 39th Nordic Meeting of Gastroenterology
www.congex.com/ngc2008
 June 6-8, Prague, Czech Republic
 3rd Annual European Meeting: Perspectives in Inflammatory Bowel Diseases
 Email: meetings@imedex.com

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

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

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

June 26-28, Bratislava, Slovakia
 5th Central European Gastroenterology Meeting
www.ceurgem2008.cz
 September 10-13, Budapest, Hungary

11th World Congress of the International Society for Diseases of the Esophagus
 Email: isde@isde.net

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

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

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

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

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

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



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

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

October 22-25, Brisbane, Australia
 Australian Gastroenterology Week 2008
 Email: gesa@gesa.org.au

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

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

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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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H. pylori recurrence after successful eradication

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Abstract

Recurrence of *H. pylori* after eradication is rare in developed countries and more frequent in developing countries. Recrudescence (recolonization of the same strain within 12 mo after eradication) rather than reinfection (colonization with a new strain, more than 12 mo after eradication) is considered to be responsible for most of the cases. This observation was confirmed only in developed countries, while in developing countries a recent meta-analysis demonstrated a high rate of reinfection. The proportion of *H. pylori* annual recurrence was 2.67% and 13.00% in developed and developing countries, respectively. Nested meta-analysis (only cases with a longer follow-up and a negative ¹³CUBT a year after eradication) revealed annual recurrence rate of 1.45% [relative risk (RR), 0.54] and 12.00% (RR, 0.92) in developed and developing countries, respectively. These findings support the notion that in developed countries many cases of recurrence are due to recrudescence within the first year after eradication, with a 46% drop in the recurrence rate after the first year post eradication, while in developing countries reinfection is more pronounced, and continue at the same rate since eradication. A different approach for follow-up after *H. pylori* eradication is probably needed in patients of developing countries, since reinfection is highly prevalent.

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Key words: *Helicobacter pylori*; Eradication; Recurrence; Recrudescence; Reinfection

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Niv Y. *H. pylori* recurrence after successful eradication. *World J Gastroenterol* 2008; 14(10): 1477-1478 Available from: URL: <http://www.wjgnet.com/1007-9327/14/1477.asp> DOI: <http://dx.doi.org/10.3748/wjg.14.1477>

A definite cure of peptic disease and prevention of ulcer complications, as well as cure of mucosa-associated lymphoid tissue (MALT) lymphoma, is dependent on successful eradication of *H. pylori*. Thus, recurrence of infection should be taken seriously into consideration.

More than 120 studies were published in the medical literature about recurrence of *H. pylori* till today, and found a wide range of recurrence rates^[1-5]. The 12-mo recurrence rate varies in different studies, from 0% to 41.5% or more^[5]. In a recent 7-year follow up study the annual recurrence rate of *H. pylori* infection after a successful eradication in Israel was only 0.55%^[6]. This may be explained by the fact that studies are extremely different in design, diagnostic methods, and population base.

Recurrence of *H. pylori* after a successful eradication is rare in developed countries and more frequent in developing countries^[1]. Recrudescence (recolonization of the same strain) rather than reinfection (colonization with a new strain) is considered more likely to be responsible for most of the cases^[5]. But this belief is based on heterogeneous methods, using different approaches. Recrudescence is a clinical problem, a result of treatment failure. Reinfection is considered a problem of preventive medicine, and should be dealt in a different way. An accurate diagnosis is difficult, and mostly relies on molecular fingerprinting techniques, confirming that the identified bacteria, before and after therapy, are genetically identical^[1]. Using this strategy it was found that recurrence was due to recrudescence in up to 80% of the cases^[7]. Nevertheless, the possibility that reinfection with a strain common to family members or another close contact, cannot be ruled out. The case may be even more complicated since different strains may be sometimes isolated from the same host^[8], and microevolution can be observed at a high frequency. Recrudescence is most likely to occur during the first year after eradication, while reinfection may account for recurrence after a year from the eradication therapy. Heavy contamination of the environment and sources such as in drinking water, institutionalized patients, medical personnel or family members, may be the source of reinfection, especially in developing countries^[7].

H. pylori infection and recurrence examined with many laboratory methods, such as ^{13}C -urea breath test ($^{13}\text{CUBT}$), ^{14}C -urea breath test, stool antigen test, urease test, histology or culture. In addition, successful eradication measured by a negative test in different periods after the treatment. While most authorities believe that 4 wk time span is enough to confirm eradication^[9], some investigators believe that this time should oscillate between 10 wk and 14 wk^[1]. A recent meta-analysis^[10] overcame the bias of changing approaches and different strategies, including 17 papers that used $^{13}\text{CUBT}$ in adults, with a minimum follow up of 12 mo^[2-4,11-24]. In addition, studies that examined *H. pylori* recurrence after a negative $^{13}\text{CUBT}$, at least a year post eradication treatment, were looked at separately. The proportion of *H. pylori* annual recurrence was 2.67% and 13.00% in developed and developing countries, respectively. Nested meta-analysis (only cases with a longer follow-up and a negative $^{13}\text{CUBT}$ a year after eradication) revealed annual recurrence rate of 1.45% [relative risk (RR), 0.54] and 12.00% (RR, 0.92) in developed and developing countries, respectively. These findings support the notion that in developed countries many cases of recurrence are due to recrudescence within the first year after eradication, with a 46% drop in the recurrence rate after the first year post eradication, while in developing countries reinfection is more pronounced, and continue at the same rate since eradication.

A different approach for follow-up after *H. pylori* eradication is probably needed in patients of developing countries, since reinfection is highly prevalent.

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Staging accuracy of esophageal cancer by endoscopic ultrasound: A meta-analysis and systematic review

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Abstract

AIM: To evaluate the accuracy of endoscopic ultrasound (EUS) in the staging of esophageal cancer.

METHODS: Only EUS studies confirmed by surgery were selected. Articles were searched in Medline and Pubmed. Two reviewers independently searched and extracted data. Meta-analysis of the accuracy of EUS was analyzed by calculating pooled estimates of sensitivity, specificity, likelihood ratios, and diagnostic odds ratio. Pooling was conducted by both the Mantel-Haenszel method (fixed effects model) and DerSimonian Laird method (random effects model). The heterogeneity of studies was tested using Cochran's *Q* test based upon inverse variance weights.

RESULTS: Forty-nine studies ($n = 2558$) which met the inclusion criteria were included in this analysis. Pooled sensitivity and specificity of EUS to diagnose T1 was 81.6% (95% CI: 77.8-84.9) and 99.4% (95% CI: 99.0-99.7), respectively. To diagnose T4, EUS had a pooled sensitivity of 92.4% (95% CI: 89.2-95.0) and specificity of 97.4% (95% CI: 96.6-98.0). With Fine Needle Aspiration (FNA), sensitivity of EUS to diagnose N stage improved from 84.7% (95% CI: 82.9-86.4) to 96.7% (95% CI: 92.4-98.9). The *P* value for the χ^2 test of heterogeneity for all pooled estimates was > 0.10 .

CONCLUSION: EUS has excellent sensitivity and specificity in accurately diagnosing the TN stage of esophageal cancer. EUS performs better with advanced (T4) than early (T1) disease. FNA substantially improves the sensitivity and specificity of EUS in evaluating N stage disease. EUS should be strongly considered for staging esophageal cancer.

INTRODUCTION

Esophageal cancer is a devastating disease with a significant impact on patients' lives and health-care systems world-wide. Esophageal cancer affects 1%-2% of people in the United States and up to 15% of people undergoing endoscopy for gastroesophageal reflux disease (GERD)^[1]. The incidence of esophageal cancer is increasing in the USA, approximately 20.6% on average annually, despite a decrease in esophageal squamous cell cancer^[2,3]. This increase is mostly due to a dramatic rise in esophageal adenocarcinoma, from 1.8 cases per 100 000 during 1987-1992 to 2.5 cases per 100 000 during 1992-1996^[4]. From 1973 to 2002, esophageal adenocarcinoma has increased fourfold^[5]. The impact of this disease is significant throughout the world due to its increasing incidence and significant mortality (5-year mortality rate $> 80\%$)^[6].

Based upon the increasing incidence and devastating consequences of esophageal adenocarcinoma, an increasing amount of resources has been evaluated and implemented in an effort to stage and treat this disease. Based upon the 1996 US national cancer database, the 5-year survival rate for esophageal cancer is as follows: stage 0 (TisN0M0) is 52%, stage I (T1N0M0) is 42%, stage II (T2N0M0 or T3N0M0) or (T1N1M0 or T2N1M0) is 29%, stage III (T3N1M0 or T4NxM0) is 15%, and stage IV (TxNxM1) is 3%^[7].

Staging of esophageal cancer is extremely important since it helps differentiate treatment options. To improve survival, many treatment modalities have been utilized for esophageal cancer, including surgery, radiotherapy, chemotherapy, and combinations of the aforementioned options^[7]. For early disease, recent studies that have investigated endoscopic mucosal resection have shown a 5-year survival of 98%^[8] and a low recurrence rate^[9]. Although multiple treatment regimens exist and they overlap

for each stage, the stage of disease is very important in guiding treatment and predicting outcomes.

Many staging modalities have been utilized for esophageal cancer, including chest CT, MRI, positron emission tomography (PET), and endoscopic ultrasound (EUS). CT and MRI lack the ability to differentiate layers of the esophageal mucosa. Thus, these modalities cannot accurately discern T stage of esophageal cancer. Chest CT provides important information regarding tumor size, lymph node involvement, and potential metastatic lesions. However, chest CT alone has a sensitivity of only 48% for mediastinal lymph node involvement^[10]. MRI has been shown to be useful in preoperative evaluation and equally as accurate as CT in staging esophageal cancer; however, studies do vary^[11]. MRI staging has been shown to have an accuracy of 40% with very low sensitivity and specificity^[12,13]. For mediastinal lymph node involvement, thoracoscopic procedures for tissue biopsy carry a risk of complications in 25%-35% of cases^[14,15]. An alternative to CT or MRI is PET. PET is a non-invasive test which has been shown to be beneficial in detection of metastatic disease (stage IV); however, detection of locoregional metastases is limited^[13]. Due to limitations of CT, MRI, and PET, other modalities, such as EUS, have been initiated and reviewed.

EUS utilizes an echoendoscope that is passed directly into the esophagus, with the ability to visualize the individual histological layers of the esophagus^[16]. This approach is particularly useful in evaluating invasion of local disease, especially esophageal cancer. EUS has been shown to detect more locoregional node involvement than CT or PET, with a higher sensitivity^[17,18]. The accuracy of EUS to determine tumor depth has also been estimated to be quite accurate^[18-20]. However, studies vary as to the accuracy of EUS in both the depth of local disease, nodal involvement, and the detection of distant metastases^[21-24].

With EUS emerging as a very useful staging tool, its role in staging esophageal cancer continues to be addressed. Several studies have identified the potential benefits of EUS with esophageal cancer staging; however, results regarding the extent of its benefits have been inconsistent^[52,72,82]. We conducted a meta-analysis to examine the role of EUS in the staging of esophageal cancer for loco-regional spread.

This meta-analysis and systematic review was written in accordance with the proposal for reporting by the QUOROM (Quality of Reporting of Meta-analyses) statement^[25]. Since this study investigated diagnostic accuracy of a test, the study design for this meta-analysis and systematic review conformed to the guidelines of the Standards for Reporting of Diagnostic Accuracy (STARD) initiative^[26].

MATERIALS AND METHODS

Study selection criteria

Only EUS studies confirmed by surgery or appropriate follow-up were selected. EUS criteria used for T staging were: T1, tumor invades the lamina propria or submucosa but not the muscularis propria; T2, tumor invades but does not extend beyond the muscularis propria; T3, tumor invades the peri-esophageal tissues but not adjacent organs;

and T4, tumor invades adjacent structures. Nodal invasion was defined as invasion of mediastinal lymph nodes. From this pool, only studies from which a 2×2 table could be constructed for true-positive, false-negative, false-positive and true-negative values were included.

Data collection and extraction

Articles were searched in Medline, Pubmed, Ovid journals, CINAHL, ACP Journal Club, DARE, International Pharmaceutical Abstracts, Old Medline, Medline Non-indexed Citations, OVID Healthstar, and Cochrane Controlled Trials Registry. The search terms used were endoscopic ultrasound, EUS, ultrasound, endosonography, esophageal cancer, esophageal cancer, tumor staging, nodal invasion, staging, surgery, sensitivity, specificity, positive predictive value, and negative predictive value. 2×2 tables were constructed with the data extracted from each study. Two authors (SP and JR) independently searched and extracted the data. Any differences were resolved by mutual agreement.

Quality of studies

Clinical trial with a control arm can be assessed for the quality of the study. A number of criteria have been used to assess this quality of a study (e.g. randomization, selection bias of the arms in the study, concealment of allocation, and blinding of outcome)^[27,28]. There is no consensus on how to assess studies without a control arm. Hence, these criteria do not apply to studies without a control arm^[28]. Therefore, for this meta-analysis and systematic review, studies were selected based on completeness of data and inclusion criteria.

Statistical analysis

Meta-analysis for the accuracy of EUS in diagnosing the etiology of mediastinal lymphadenopathy was performed by calculating pooled estimates of sensitivity, specificity, likelihood ratios, and diagnostic odds ratios. EUS studies were grouped into periods of time to standardize the change in EUS technology and EUS criteria for lymph node involvement^[29]. These periods of time were 1986-1994, 1995-1999 and 2000-2006. Pooling was conducted using the Mantel-Haenszel method (fixed effects model) and DerSimonian Laird method (random effects model). The confidence intervals (CIs) were calculated using the F distribution method^[30]. Forrest plots were drawn to show the point estimates in each study, in relation to the summary pooled estimate. The width of the point estimates in the Forrest plots indicated the assigned weight for that study. For 0 values, 0.5 was added, as described by Cox^[31]. The heterogeneity of the sensitivities and specificities was tested by applying the likelihood ratio test^[32]. The heterogeneity of likelihood ratios and diagnostic odds ratios were tested using Cochran's Q test, based upon inverse variance weights^[33]. Heterogeneity among studies was also tested by using summary receiver operating characteristic (SROC) curves. SROC curves were used to calculate the area under the curve (AUC). The effect of publication and selection bias on the summary estimates was tested by the Egger^[34] and

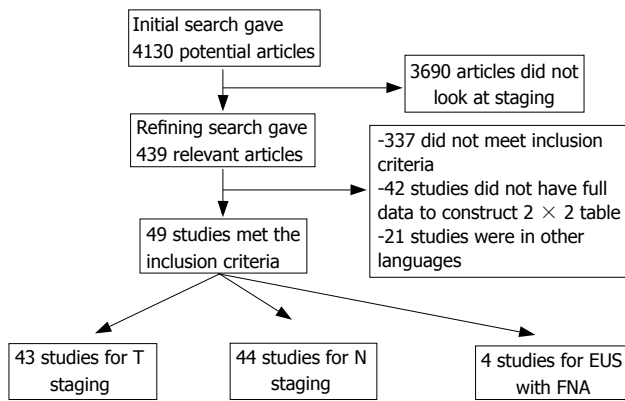


Figure 1 Search results.

Begg-Mazumdar^[35] bias indicators. Also, funnel plots were constructed to evaluate potential publication bias using the standard error and diagnostic odds ratio^[36,37].

RESULTS

An initial search identified 4130 reference articles, of these, 439 relevant articles were selected and reviewed. Forty-nine studies ($n = 2558$) which met the inclusion criteria were included in this analysis^[10,18,20-24,38-40]. For T staging, there were 43 studies^[10,18,20-24,39-72]. There were 44 studies for nodal staging^[10,18,20-24,38-46,48-50,53,54,56-63,66-80], and of these, 4 used FNA for nodal staging^[23,38,76,77]. Figure 1 shows the search results and Table 1 the characteristics for EUS studies included in this meta-analysis. All of the 49 studies included were published as full-text articles in peer-review journals. Not all studies had data for all the stages; we only used data for the available stage of esophageal cancer in a given paper. All the studies included used dedicated EUS machines. The calculated pooled estimates given are estimates calculated by the fixed effect model.

Accuracy of EUS for T staging

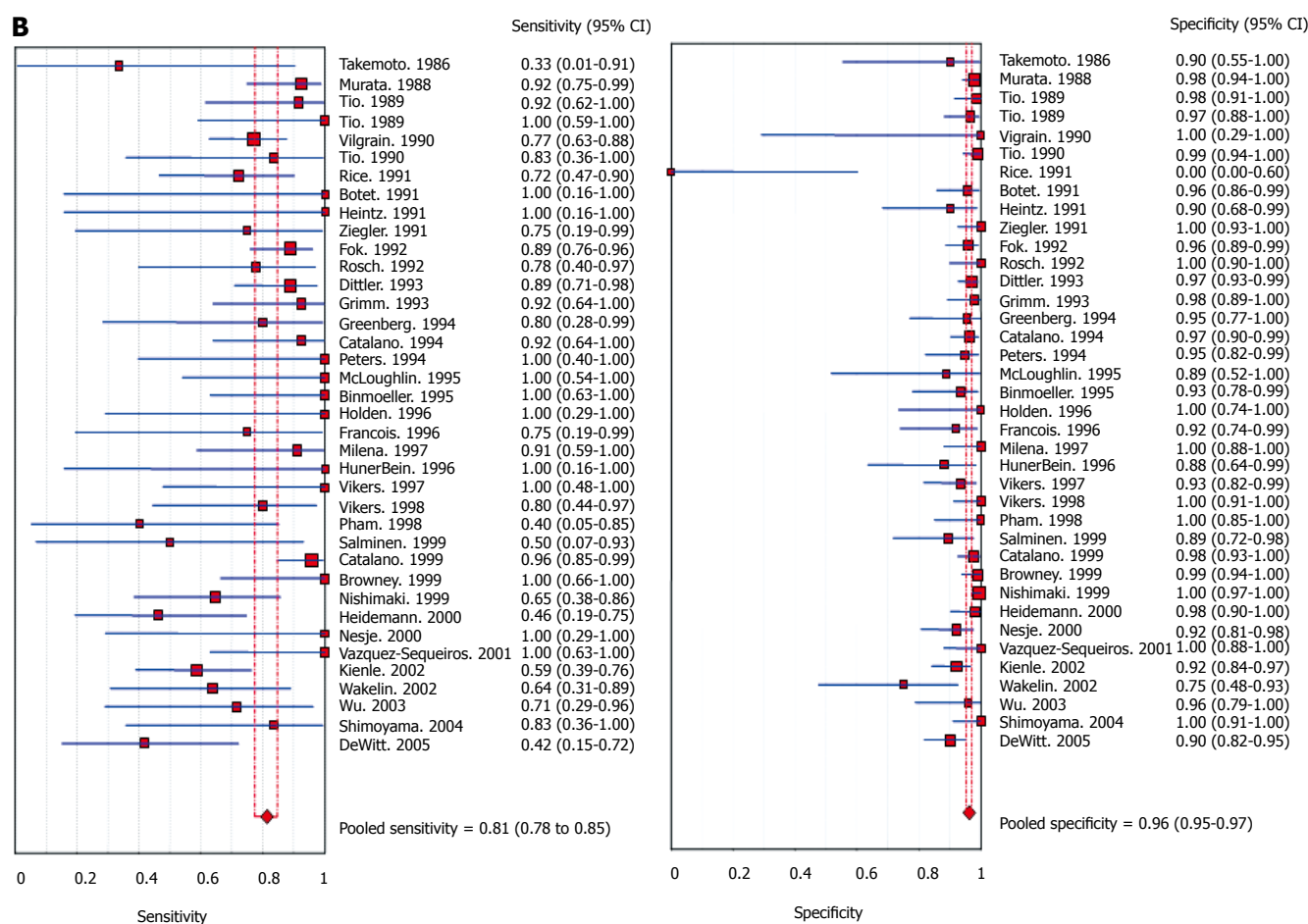
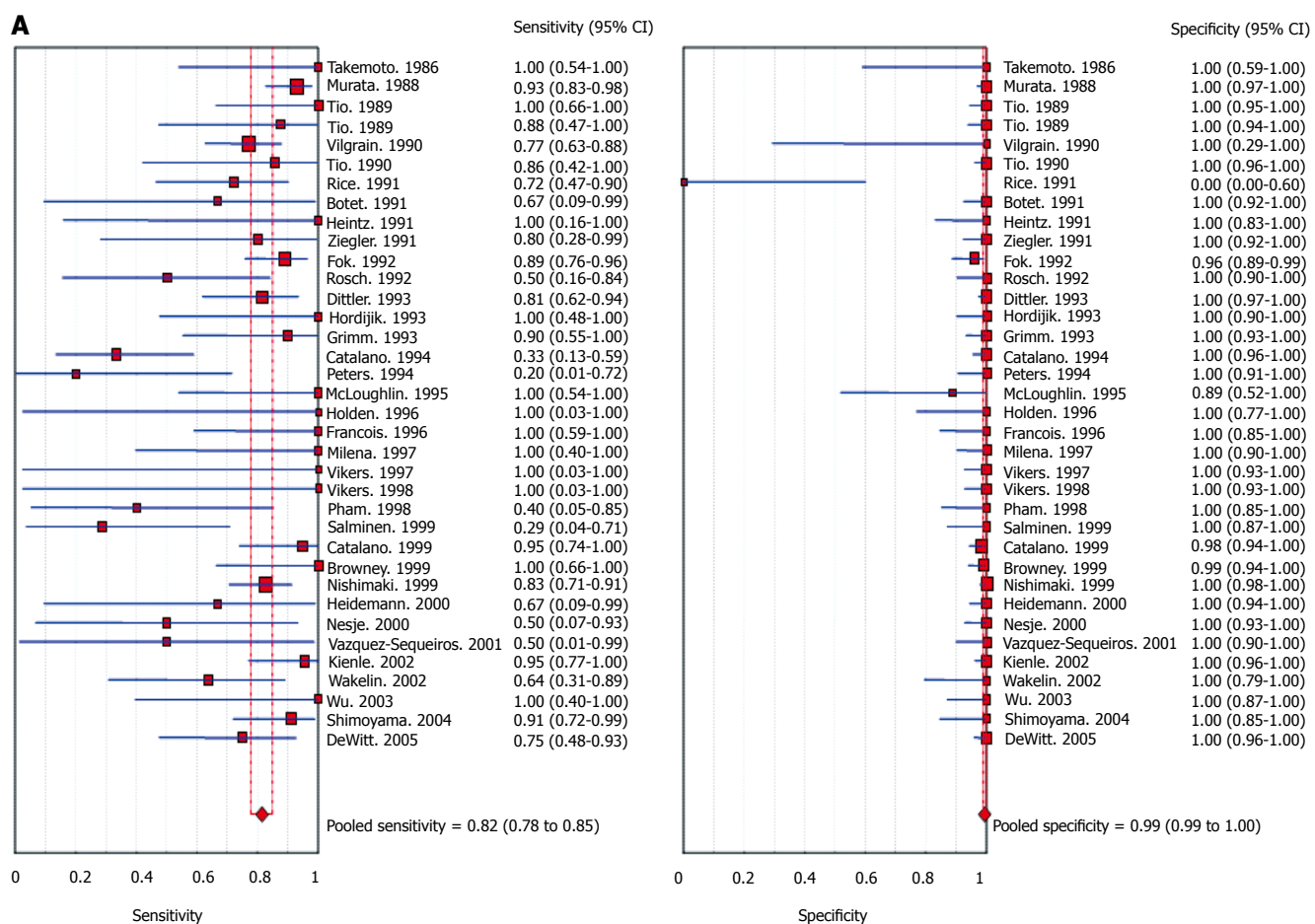
The pooled sensitivity and specificity of EUS to diagnose T1 stage cancer was 81.6% (95% CI: 77.8-84.9) and 99.4% (95% CI: 99.0-99.7), respectively. Figure 2A shows the sensitivity and specificity to diagnose T1 stage cancer in a Forrest plot. For T2 stage, EUS had a pooled sensitivity and specificity of 81.4% (95% CI: 77.5-84.8) and 96.3% (95% CI: 95.4-97.1), respectively. The Forrest plot in Figure 2B shows the sensitivity and specificity of EUS to diagnose T2 stage cancer. For T3 stage, EUS had a pooled sensitivity and specificity of 91.4% (95% CI: 89.5-93.0) and 94.4% (95% CI: 93.1-95.5), respectively. Figure 2C shows the ability of EUS to diagnose stage T3. To diagnose T4 stage cancer, EUS had a pooled sensitivity of 92.4% (95% CI: 89.2-95.0) and specificity of 97.4% (95% CI: 96.6-98.0). The sensitivity and specificity of EUS to diagnose T4 stage cancer from individual studies are shown as a Forrest plot in Figure 2D. A test of heterogeneity for all the pooled estimates for T stages had a P value > 0.10 . All the pooled estimates calculated by fixed and random effect models were similar. Table 2 shows the pooled accuracy estimates of EUS for T stage esophageal cancer.

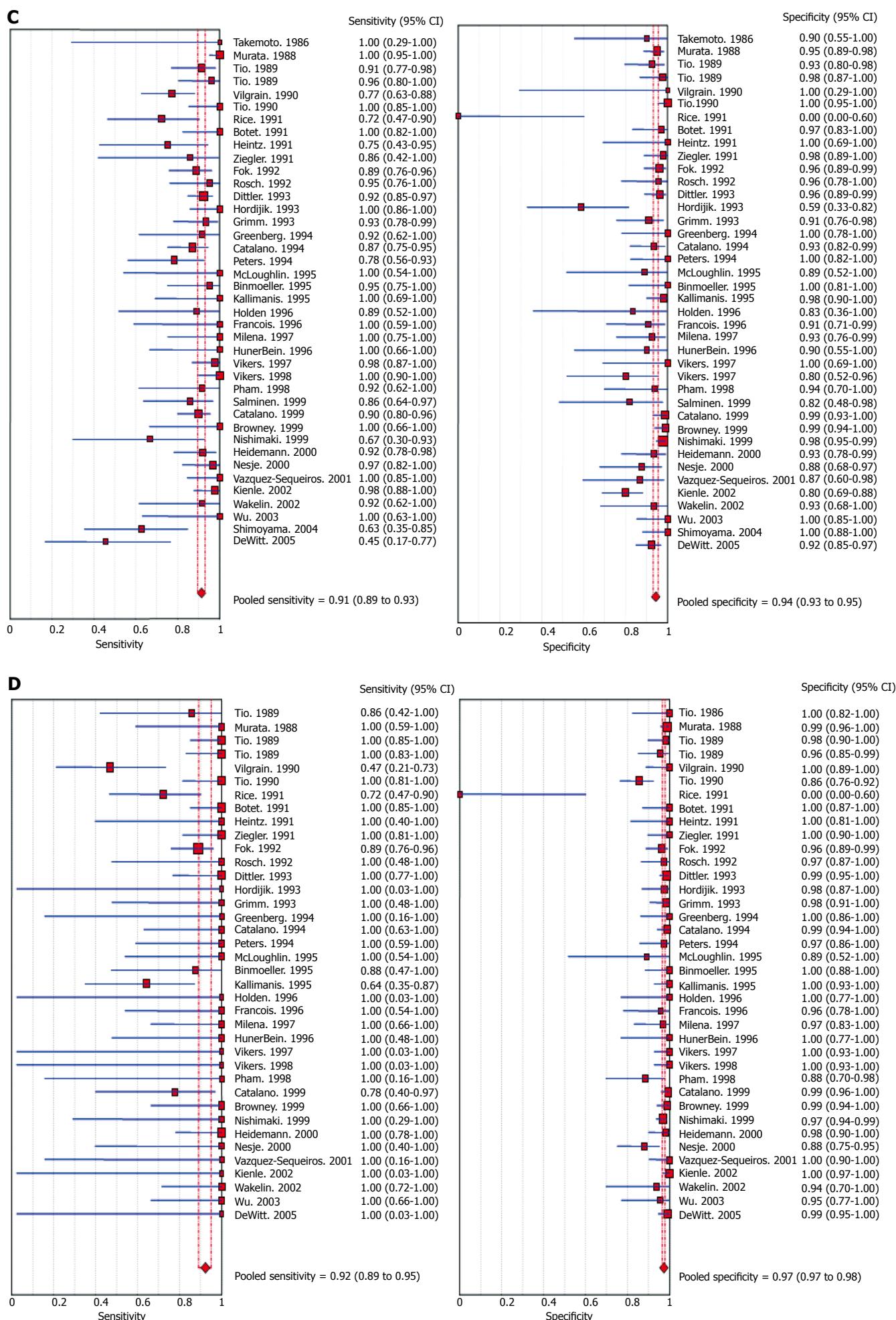
Table 1 Characteristics of studies included in this analysis

	Author	Year of publication	Type of enrolment	Confirmatory test
1	Takemoto <i>et al</i>	1986	Consecutive	Surgery
2	Tio <i>et al</i>	1986	Prospective	Surgery
3	Murata <i>et al</i>	1988	Consecutive	Surgery
4	Tio <i>et al</i>	1989	Prospective	Surgery
5	Vilgrain <i>et al</i>	1990	Consecutive	Surgery
6	Botet <i>et al</i>	1991	Consecutive	Surgery
7	Tio <i>et al</i>	1989	Prospective	Surgery
8	Heintz <i>et al</i>	1991	Consecutive	Surgery
9	Rice <i>et al</i>	1991	Consecutive	Surgery
10	Ziegler <i>et al</i>	1991	Consecutive	Surgery
11	Tio <i>et al</i>	1990	Consecutive	Surgery
12	Fok <i>et al</i>	1992	Consecutive	Surgery
13	Rosch <i>et al</i>	1992	Consecutive	Surgery
14	Dittler <i>et al</i>	1993	Consecutive	Surgery
15	Grimm <i>et al</i>	1993	Prospective	Surgery
16	Hordijk <i>et al</i>	1993	Consecutive	Surgery
17	Yoshikane <i>et al</i>	1993	Consecutive	Surgery
18	Catalano <i>et al</i>	1994	Consecutive	Surgery
19	Greenberg <i>et al</i>	1994	Prospective	Surgery
20	Peters <i>et al</i>	1994	Consecutive	Surgery
21	Binmoeller <i>et al</i>	1995	Prospective	Surgery
22	Kallimanis <i>et al</i>	1995	Consecutive	Surgery
23	McLoughlin <i>et al</i>	1995	Consecutive	Surgery
24	Francois <i>et al</i>	1996	Consecutive	Surgery
25	Hasegawa <i>et al</i>	1996	Consecutive	Surgery
26	Holden <i>et al</i>	1996	Consecutive	Surgery
27	Hunerbein <i>et al</i>	1996	Consecutive	Surgery
28	Massari <i>et al</i>	1996	Prospective	Surgery
29	Natsugoe <i>et al</i>	1996	Consecutive	Surgery
30	Vikers <i>et al</i>	1997	Consecutive	Surgery
31	Shimizu <i>et al</i>	1997	Consecutive	Surgery
32	Pham <i>et al</i>	1998	Consecutive	Surgery
33	Vikers <i>et al</i>	1998	Prospective	Surgery
34	Browney <i>et al</i>	1999	Prospective	Surgery
35	Catalano <i>et al</i>	1999	Prospective	Surgery
36	Nishimaki <i>et al</i>	1999	Consecutive	Surgery
37	Salminen <i>et al</i>	1999	Consecutive	Surgery
38	Giovannini <i>et al</i>	1999	Prospective	Surgery
39	Krasna <i>et al</i>	1999	Consecutive	Surgery
40	Heidemann <i>et al</i>	2000	Consecutive	Surgery
41	Nesje <i>et al</i>	2000	Prospective	Surgery
42	Vazquez-Sequeiros <i>et al</i>	2001	Consecutive	Surgery
43	Wiersema <i>et al</i>	2001	Prospective	Surgery
44	Kienle <i>et al</i>	2002	Prospective	Surgery
45	Wakelin <i>et al</i>	2002	Consecutive	Surgery
46	Schwartz <i>et al</i>	2002	Consecutive	Surgery
47	Wu <i>et al</i>	2003	Prospective	Surgery
48	Shimoyama <i>et al</i>	2004	Consecutive	Surgery
49	DeWitt <i>et al</i>	2005	Prospective	Surgery

Accuracy of EUS for N staging

With FNA, the sensitivity of EUS to diagnose N stage cancer improved from 84.7% (95% CI: 82.9-86.4) to 96.7% (95% CI: 92.4-98.9). Figure 2E depicts the sensitivity of EUS alone and EUS with FNA in diagnosing N stage cancer. The specificity of EUS improved from 84.6% (95% CI: 83.2-85.9) to 95.5% (95% CI: 91.0-98.2) with FNA. The Forrest plot in Figure 2F shows the specificity of EUS alone and EUS with FNA in diagnosing nodal invasion by esophageal cancer. The accuracy estimates of EUS alone and EUS with FNA are shown in Table 3. All the pooled estimates calculated by fixed and random effect models were similar. The P values for χ^2 heterogeneity for all the pooled accuracy estimates were > 0.10 .





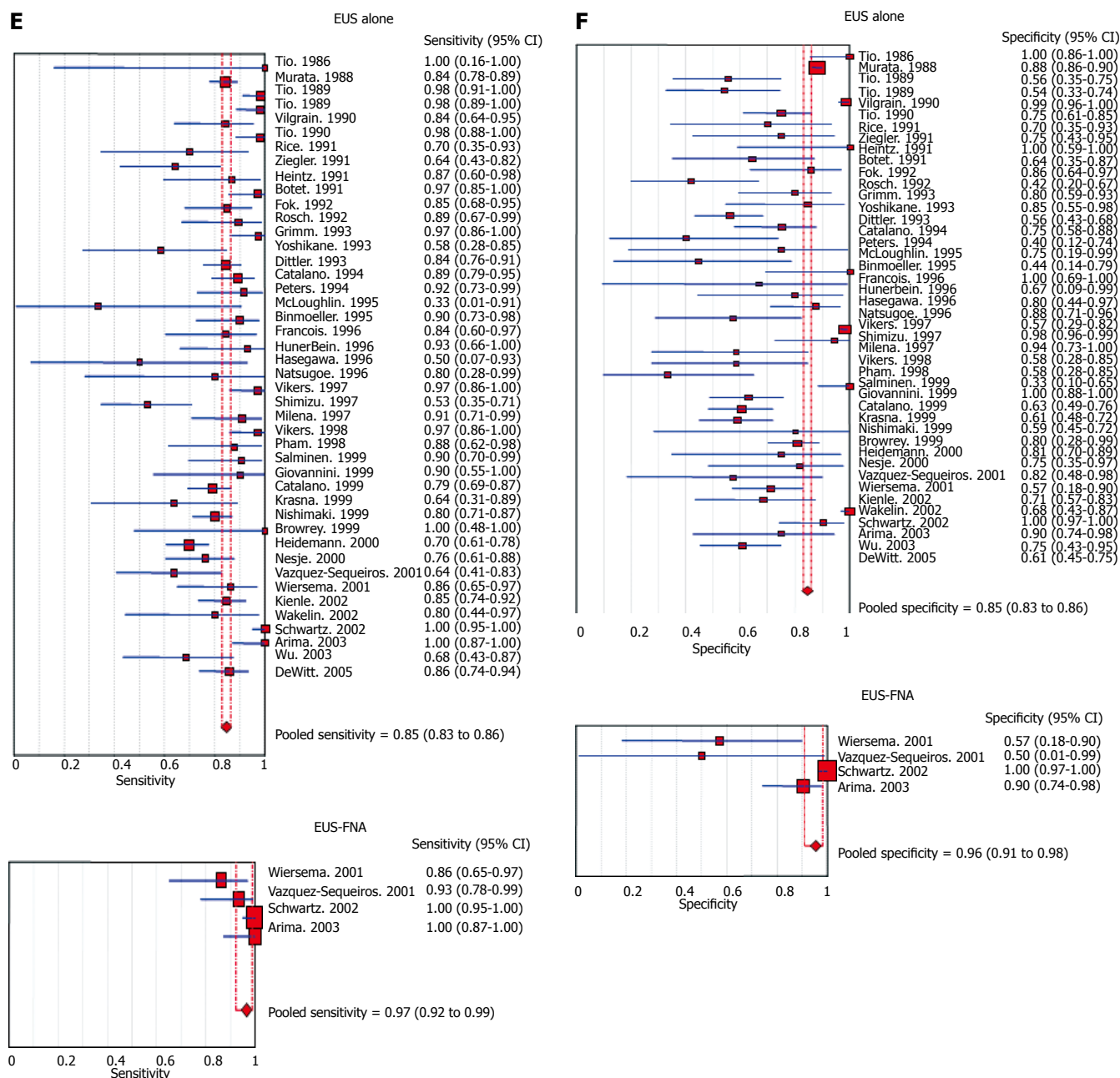


Figure 2 A: Forrest plot showing sensitivity and specificity of EUS to diagnose T1 stage of esophageal cancer; B: Forrest plot showing sensitivity and specificity of EUS to diagnose T2 stage of esophageal cancer; C: Forrest plot showing sensitivity and specificity of EUS to diagnose T3 stage of esophageal cancer; D: Forrest plot showing sensitivity and specificity of EUS to diagnose T4 stage of esophageal cancer; E: Forrest plot showing sensitivity of EUS alone and EUS with FNA for N staging of esophageal cancer; F: Forrest plot showing specificity of EUS alone and EUS with FNA for N staging of esophageal cancer.

Table 2 Accuracy of EUS with CIs to diagnose T stage in esophageal cancer

	Pooled sensitivity (%)	Pooled specificity (%)	Pooled LR+	Pooled LR-	Pooled DOR
T1	81.6 (77.8-84.9)	99.4 (99.0-99.7)	44.4 (15.5-127.4)	0.2 (0.2-0.4)	221.5 (118.5-413.9)
T2	81.4 (77.5-84.8)	96.3 (95.4-97.1)	16.6 (9.3-29.7)	0.2 (0.2-0.3)	90.7 (48.3-170.5)
T3	91.4 (89.5-93.0)	94.4 (93.1-95.5)	12.5 (7.7-20.3)	0.1 (0.1-0.2)	145.2 (90.3-233.4)
T4	92.4 (89.2-95.0)	97.4 (96.6-98.0)	25.4 (13.7-47.0)	0.1 (0.1-0.2)	250.0 (145.2-430.5)

LR+: Positive likelihood ratio; LR-: Negative likelihood ratio; DOR: Diagnostic odds ratio.

Table 3 Pooled estimate of accuracy of EUS alone and EUS-FNA in nodal staging of esophageal cancer with 95% CIs

	EUS	EUS-FNA
Studies	44	4
Pooled sensitivity (%)	84.7 (82.9-86.4)	96.7 (92.4-98.9)
Pooled specificity (%)	84.6 (83.2-85.9)	95.5 (91.0-98.2)
Positive likelihood ratio	3.3 (2.6-4.3)	7.3 (0.9-54.3)
Negative likelihood ratio	0.24 (0.9-0.3)	0.05 (0.01-0.64)
Diagnostic odds ratio	19.1 (12.7-28.5)	164.5 (4.5-6027.7)

Effect of technology

EUS studies were grouped into three periods of time

Table 4 Accuracy of EUS with CIs to stage esophageal cancer over the past two decades

	Year	No. of studies	Pooled sensitivity (%)	Pooled specificity (%)	Pooled LR +	Pooled LR-	Pooled DOR
T1	1986-1944	17	80.4 (75.2-84.8)	99.2 (98.4-99.7)	41.5 (6.1-283.3)	0.25 (0.14-0.43)	181.9 (60.7-545.7)
	1995-1999	11	83.9 (76.0-90.0)	99.4 (98.4-99.8)	36.4 (18.5-71.6)	0.21 (0.09-0.47)	299.9 (107.8-834.1)
	2000-2006	8	82.4 (72.6-89.8)	100.0 (99.1-100.0)	59.5 (22.0-161.1)	0.27 (0.16-0.47)	261.2 (81.4-838.0)
T2	1986-1994	17	85.2 (80.2-89.4)	96.8 (95.5-97.8)	18.6 (5.9-58.6)	0.19 (0.12-0.30)	123.9 (47.7-322.0)
	1995-1999	13	86.8 (79.7-92.1)	97.4 (95.8-98.5)	16.9 (9.1-31.1)	0.20 (0.11-0.38)	139.5 (56.6-343.8)
	2000-2006	8	62.9 (52.0-72.9)	93.4 (90.4-95.6)	8.3 (4.3-15.9)	0.47 (0.34-0.64)	24.7 (9.1-67.4)
T3	1986-1994	18	90.8 (88.1-93.0)	94.6 (92.6-96.2)	13.9 (5.2-36.9)	0.12 (0.07-0.19)	157.7 (70.9-351.1)
	1995-1999	14	93.7 (90.0-96.3)	96.4 (94.5-97.7)	12.6 (7.6-20.9)	0.11 (0.08-0.17)	159.4 (77.9-326.2)
	2000-2006	8	89.9 (84.5-93.9)	90.0 (86.1-93.2)	7.0 (4.6-10.8)	0.11 (0.04-0.32)	100.9 (33.5-303.9)
T4	1986-1994	18	92.1 (87.9-95.2)	96.9 (95.6-97.9)	24.7 (8.4-72.7)	0.09 (0.04-0.23)	278.8 (97.2-799.9)
	1995-1999	14	89.2 (79.8-95.2)	98.0 (96.7-98.96)	22.2 (13.2-37.3)	0.23 (0.15-0.36)	227.1 (89.7-575.0)
	2000-2006	8	100.0 (91.8-100.0)	97.5 (95.4-98.8)	20.2 (8.8-46.3)	0.11 (0.04-0.29)	272.6 (73.4-1013.2)
N	1986-1994	17	88.0 (85.4-90.2)	85.2 (83.4-86.9)	3.6 (2.4-5.4)	0.2 (0.1-0.3)	27.6 (14.6-52.4)
	1995-1999	17	82.6 (78.0-85.9)	84.4 (81.6-86.9)	3.0 (2.1-4.5)	0.3 (0.2-0.4)	14.8 (7.5-29.3)
	2000-2005	10	81.6 (77.8-85.1)	82.4 (78.2-86.1)	3.4 (2.2-5.3)	0.3 (0.2-0.4)	14.9 (6.7-33.1)

Table 5 Bias indicators and AUC with the corresponding Q values for various cancer stages

	Begg-Mazumdar bias (Kendall's tau value, <i>P</i>)	Egger bias (95% CI, <i>P</i>)	AUC (SE)	Q (SE)
T1	-0.51, <i>P</i> = 0.01	-0.48 (95% CI = -2.84 to 1.88, <i>P</i> = 0.68)	0.97 (0.02)	0.91 (0.02)
T2	-0.14, <i>P</i> = 0.24	-0.32 (95% CI = -1.74 to 1.10, <i>P</i> = 0.65)	0.95 (0.02)	0.89 (0.02)
T3	-0.11, <i>P</i> = 0.32	0.33 (95% CI = -1.43 to 2.09, <i>P</i> = 0.70)	0.97 (0.01)	0.92 (0.01)
T4	-0.07, <i>P</i> = 0.56	-2.89 (95% CI = -5.35 to -0.44, <i>P</i> = 0.02)	0.98 (0.01)	0.93 (0.01)
N	-0.26, <i>P</i> = 0.01	0.29 (95% CI = -1.58 to 1.00, <i>P</i> = 0.69)	0.91 (0.02)	0.99 (0.02)

to standardize the change in EUS technology and the change in EUS criteria for tumor staging. These periods were 1986-1994, 1995-1999 and 2000-2006. The pooled estimates of studies during these periods of time are shown in Table 4. The *P* value for χ^2 heterogeneity for all the pooled accuracy estimates was > 0.10 .

Bias estimates

The publication bias calculated by the Begg-Mazumdar and Egger bias indicators for each stage of esophageal cancer invasion is shown in Table 5. The funnel plots to investigate the effect of publication bias on T stage is shown in Figure 3A. The effect of publication bias on N stage is shown in Figure 3B.

SROC curves were drawn for AUC and *Q* values. The AUC and *Q* values of EUS to diagnose various stages of esophageal cancer are shown in Table 5. SROC curves for T and N staging are shown in Figure 4A and B, respectively.

A subgroup analysis was performed by removing the studies in which the last or the first author was the same (e.g. Tio *et al.*). This was done to make sure that the same data were not used by the studies, i.e. to avoid duplication. In the subgroup analysis, there was no significant change in the pooled estimates. Separate accuracy estimated for radial *versus* linear EUS technology could not be performed

as the majority of the studies did not make a distinction or give separate accuracy values for radial or linear EUS technology.

DISCUSSION

This meta-analysis and systematic review shows that the pooled sensitivity of EUS for tumor invasion (T stage) is high (about 81%-90%), with it being higher for advanced disease than early disease. For all the T stages, the pooled specificity of EUS to diagnose depth of tumor invasion is very high (about 99%). Diagnostic odds ratio is defined as the odds of having a positive test in patients with a true anatomic stage of the disease when compared to patients who do not have the disease. EUS as a diagnostic test has a very high diagnostic odds ratio for T staging (about 250 times). For example, if EUS demonstrates that a patient has T1 stage disease, the odds of having the correct anatomic stage of T1 disease is 221 to 1. This helps physicians offer endoscopic treatment with confidence to patients with early disease^[81-87]. Another way of looking at this is: if a small lesion is found to be esophageal cancer, then EUS is an excellent diagnostic test to examine the depth of tumor invasion, because of its very high sensitivity and specificity. The depth of tumor invasion can help decide if curative surgical or curative endoscopic mucosal resection or submucosal dissection can be offered to resect the lesion en bloc^[81-87].

The positive likelihood ratio of a test is a gauge of how well it identifies a disease state. The higher the positive likelihood ratio, the better the test performs in identifying the true disease status. On the other hand, a negative likelihood ratio is a gauge of how well the test performs in excluding a disease state. The lower the negative likelihood ratio, the better the test performs in excluding a disease. For T staging, EUS has a high positive likelihood ratio for all T stages and a low negative likelihood ratio for T4 disease when compared to T1 disease. This indicates that EUS performs better in excluding T4 than T1 disease. Clinically, another viewpoint is: if EUS diagnoses T2 disease then the patient might still have anatomic T1 disease, but if EUS diagnoses T1 disease then the patient probably truly has

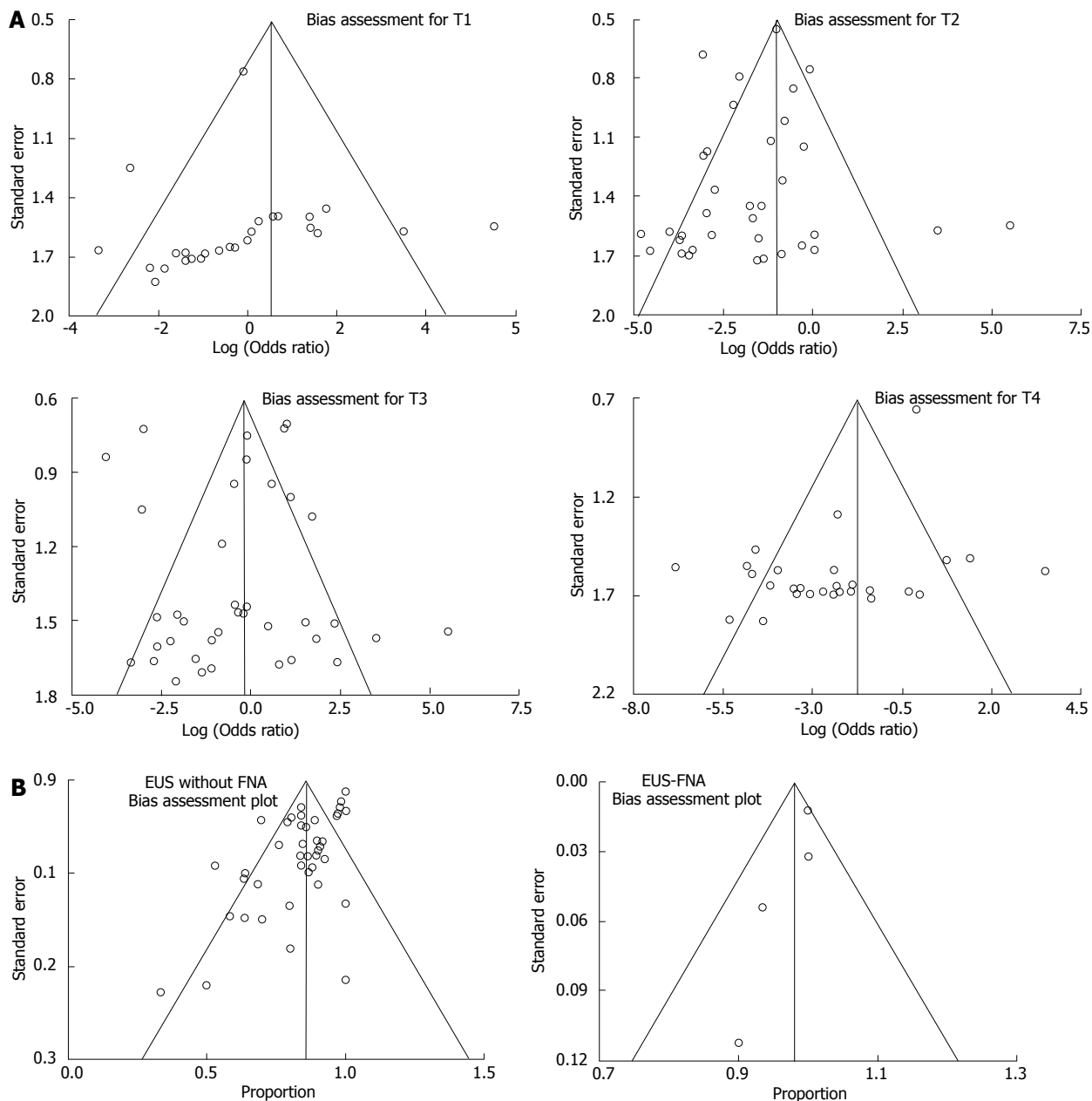


Figure 3 A: Funnel plots assessing bias for T staging; B: Funnel plots assessing bias for N staging.

anatomic T1 disease. This helps physicians offer surgical or endoscopic treatments with confidence if EUS diagnoses a patient with T1 esophageal cancer^[81-87].

The major advantage of EUS is the ability to perform FNA during the procedure for tissue diagnosis. The procedure is, in comparison with other alternative options, safe, less invasive, and does not require general anesthesia or hospitalization^[88]. The complication rate is extremely low (0.5%-2.3%), with several studies reporting no complications^[75,76,88,89]. Other modalities using FNA, such as transbronchial CT or thorascopic procedures, cannot be used for the entire mediastinum^[14,15,92-101]. EUS has the ability to image the aortopulmonary window, the subcarinal nodes, inferior mediastinum, and the entire posterior part of the mediastinum.

EUS as an imaging modality has high sensitivity and specificity to diagnose N stage esophageal cancer. This

meta-analysis shows that FNA substantially improves the sensitivity (85% to 97%) and specificity (85% to 96%) of EUS in evaluating N stage esophageal cancer, therefore, EUS with FNA should be the diagnostic test of choice.

Over the last two decades, the specificity of EUS to diagnose T stage cancer has remained high. In addition, the sensitivity of EUS for T staging has improved, especially for early disease (T1), over the past two decades, which may represent improvement in imaging technology or training. For nodal staging, all the studies in which FNA was performed were from the most recent periods. The sensitivity and specificity of EUS alone to diagnose N stage cancer has not improved in the past two decades. Our meta-analysis demonstrates that the sensitivity and specificity of EUS markedly improved with FNA.

EUS as a diagnostic tool is not designed to detect distant metastasis, so this was not evaluated in this analysis.

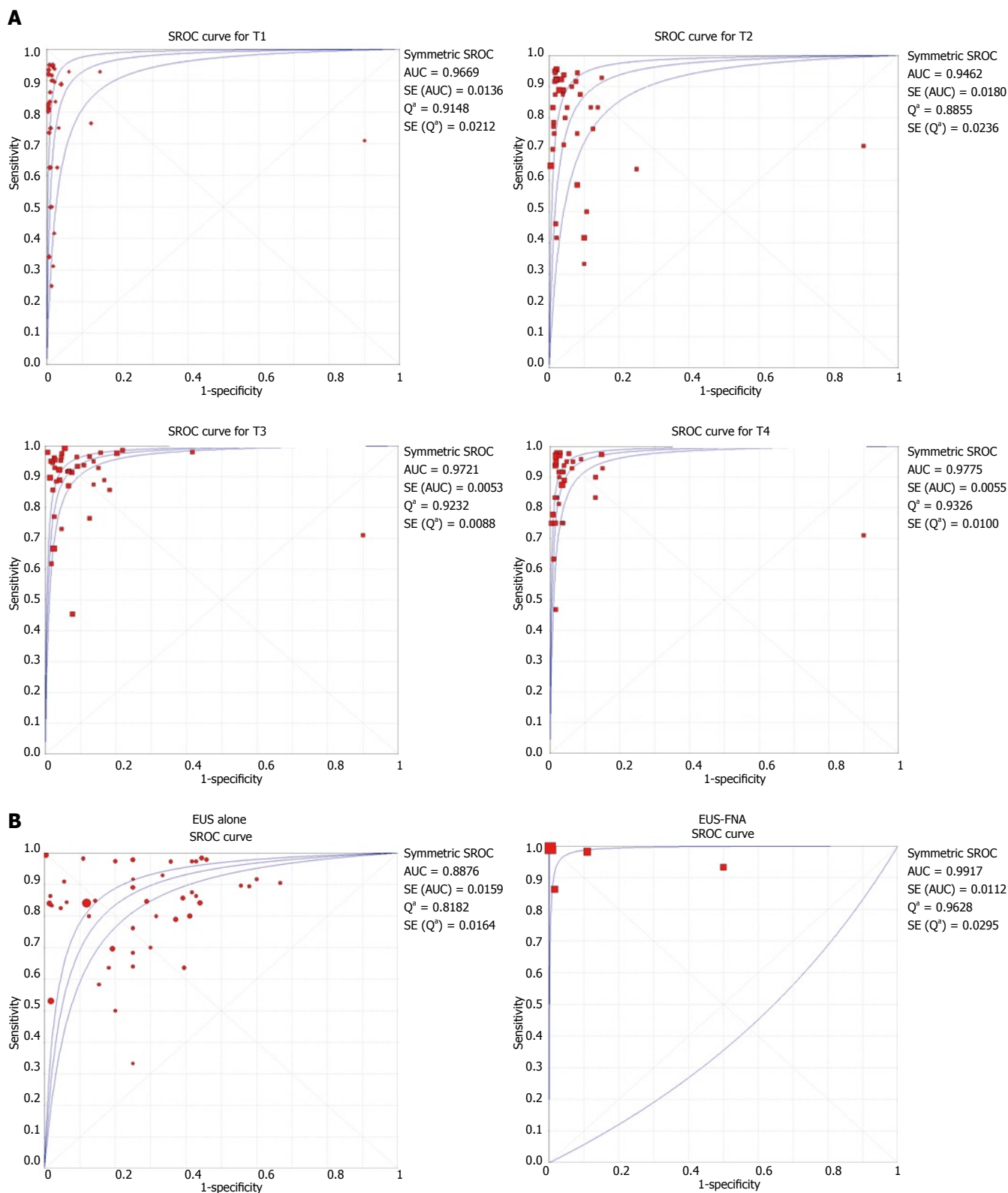


Figure 4 A: SROC curves for various T stages of esophageal cancer; B: SROC curves for various N stages of esophageal cancer.

Heterogeneity among different studies was determined by drawing SROC curves and finding the AUC, since different studies might use slightly different criteria for staging. An AUC of 1 for any test indicates that the test is excellent. SROC curves for EUS showed that AUC was very close to 1, which indicates that EUS is an excellent diagnostic test for staging esophageal cancer.

Studies with statistically significant results tend to be published and cited. Smaller studies may show larger treatment effects due to fewer case-mix differences (e.g. patients with only early or late disease) than larger trials. This can be estimated by bias indicators and construction of funnel plots. This publication and selection bias may affect the summary estimates. Also, bias among studies can

affect the shape of the funnel plot. In this meta-analysis and systematic review, bias calculations using the Egger^[35] and Begg-Mazumdar^[36] bias indicators showed no statistically significant bias. Furthermore, funnel plot analyses showed no significant bias for EUS studies.

In conclusion, EUS has excellent sensitivity and specificity in accurately diagnosing T stage esophageal cancer. EUS performs better with advanced (T4) than early (T1) disease. FNA substantially improves the sensitivity and specificity of EUS in evaluating N stage esophageal cancers. EUS should be the test of choice for TN staging of esophageal cancer.

COMMENTS

Background

Prognosis and modality of treatment in patients with esophageal cancer depends on the staging of the tumor. The published data on the accuracy of endoscopic ultrasound (EUS) for staging esophageal cancer is varied. The aim of this meta-analysis and systematic review was to evaluate the accuracy of EUS in staging esophageal cancer.

Research frontiers

To date, there have been many studies on EUS in staging esophageal cancer, but no meta-analyses.

Innovations and breakthroughs

With EUS emerging as a very useful staging tool, its role in esophageal cancer continues to be addressed. Several studies have identified the potential benefits of EUS for esophageal cancer staging; however, results regarding the extent of its benefits have been inconsistent.

Applications

EUS has excellent sensitivity and specificity in accurately diagnosing TN stage of esophageal cancer. EUS performs better with advanced disease (T4) than early disease (T1). FNA substantially improves the sensitivity and specificity of EUS in evaluating N stage cancer. EUS should be strongly considered for staging esophageal cancer.

Terminology

EUS utilizes an echoendoscope which is passed directly into the esophagus, with the ability to visualize the individual histological layers of the esophagus. This approach is particularly useful in evaluating invasion of local disease, especially in esophageal cancer.

Peer review

This manuscript is well designed and prepared study finding an important conclusions. The most important point is that the real time PCR is significantly cheaper than the other commercial test.

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Synergistic growth inhibitory effects of *Phyllanthus emblica* and *Terminalia bellerica* extracts with conventional cytotoxic agents: Doxorubicin and cisplatin against human hepatocellular carcinoma and lung cancer cells

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Abstract

AIM: To examine the growth inhibitory effects of *Phyllanthus emblica* (*P. emblica*) and *Terminalia bellerica* (*T. bellerica*) extracts on human hepatocellular carcinoma (HepG2), and lung carcinoma (A549) cells and their synergistic effect with doxorubicin or cisplatin.

METHODS: HepG2 and A549 cells were treated with *P. emblica* and *T. bellerica* extracts either alone or in combination with doxorubicin or cisplatin and effects on cell growth were determined using the sulforhodamine B (SRB) assay. The isobologram and combination index (CI) method of Chou-Talalay were used to evaluate interactions between plant extracts and drugs.

RESULTS: *P. emblica* and *T. bellerica* extracts demonstrated growth inhibitory activity, with a certain degree of selectivity against the two cancer cell lines tested. Synergistic effects (CI < 1) for *P. emblica*/

doxorubicin or cisplatin at different dose levels were demonstrated in A549 and HepG2 cells. The *T. bellerica*/cisplatin or doxorubicin also showed synergistic effects in A549 and HepG2 cells. In some instances, the combinations resulted in antagonistic effects. The dose reduction level was different and specific to each combination and cell line.

CONCLUSION: The growth inhibitory activity of doxorubicin or cisplatin, as a single agent, may be modified by combinations of *P. emblica* or *T. bellerica* extracts and be synergistically enhanced in some cases. Depending on the combination ratio, the doses for each drug for a given degree of effect in the combination may be reduced. The mechanisms involved in this interaction between chemotherapeutic drugs and plant extracts remain unclear and should be further evaluated.

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Key words: Cisplatin; Doxorubicin; Liver cancer; *Phyllanthus emblica*; Synergistic effect; *Terminalia bellerica*

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INTRODUCTION

Cancer is the third leading cause of death worldwide,

preceded by cardiovascular and infectious diseases. Doxorubicin and cisplatin represent the current standard chemotherapeutic drugs for treatment of cancers. Although there are many therapeutic strategies including chemotherapy to treat cancer, high systemic toxicity and drug resistance limit the successful outcomes in most cases. Accordingly, several new strategies are being developed to control and treat cancer. One such approach could be a combination of an effective phytochemicals with chemotherapeutic agents, which when combined, would enhance efficacy while reducing toxicity to normal tissues.

The notion has evolved that natural products frequently exert a valuable role in broadening the scope of disease intervention strategies used by drug designers. Several herbs and plants with diversified pharmacological properties are known to be rich sources of chemical constituents that may have potential for the prevention and/or treatment of several human cancers^[1-4].

P. emblica L. (Euphorbiaceae) known as emblic myrobalan, a shrub or tree, is widely used in folk medicine in Southeast Asia. This plant exhibits a variety of pharmacological effects including anti-inflammatory, anti-pyretic, anti-oxidant, anti-carcinogenic, and anti-mutagenic effects^[5-7]. The active principles or extracts of *P. emblica* have demonstrated anti-proliferative effects in several cancer cell lines both *in vitro* and *in vivo*^[7,8]. The anti-tumor activity of *P. emblica* extract was attributed to its ability to interfere with cell cycle regulation *via* the inhibition of cdc 25 phosphatase and partial inhibition of cdc 2 kinase activity^[8].

T. bellerica (Combretaceae), known as belleric myrobalan, is a large deciduous tree, common to the plains and lower hills of Southeast Asia. This plant exhibits several pharmacological effects including anti-bacterial, anti-malarial, anti-fungal, anti-HIV, anti-oxidant, and anti-mutagenic effects^[9-12]. *T. bellerica* extract exhibited anti-proliferative effects in several cancer cell lines including Shiongi 115, breast cancer MCF-7, prostate cancer PC-3 and DU-145 cells^[13]. Phytochemical studies have shown that *T. bellerica* contains a variety of chemical components, including gallic acid^[14,15]. Furthermore, recent studies have demonstrated the cytotoxic activity of gallic acid in human leukemia HK-63, HOS-1 cell, HSC-2, and HL-60 cell lines^[16-19]. Gallic acid has also been shown to induce apoptotic cell death in HSC-2 and HL-60 cells^[16,18].

These findings prompted us to test the anti-proliferative activity of *P. emblica* and *T. bellerica* extracts on human lung carcinoma A549, and human hepatocellular carcinoma HepG2 cell lines, and to investigate whether it can synergize the inhibitory action of doxorubicin and cisplatin, the standard chemotherapeutic drugs.

MATERIALS AND METHODS

Plant extracts and chemotherapeutic drugs

The dried fruits of *T. bellerica* and *P. emblica* were collected from Sraekaw and Nan Provinces in northeastern and northern Thailand, respectively. The voucher specimens were identified and kept by Associate Professor Dr. Noppamas Soonthornchareon, Department of Pharmacognosy, Faculty of Pharmacy, Mahidol University.

The dried fruits of medicinal plant were obtained and extracted with boiling water. Then, the aqueous solution was separated from the plant residues by filtration. After that, the aqueous solution was spray-dried. For testing, the extracts were dissolved in media and diluted to the desired concentrations.

Doxorubicin and Cisplatin (Platinol) were purchased from Ebewe (Austria) and Bristol-Myers Squibb (USA), respectively. The drugs were dissolved in water for injection at a concentration of 2 mg/mL. Both drugs were kept in aliquots at 4°C. Serial dilutions of these drugs were performed in culture media, immediately before each experiment, in order to obtain the required final concentrations.

Cell culture

The human lung carcinoma (A549) cell was kindly provided by the Chulabhorn Research Institute. The human hepatocellular carcinoma (HepG2) cell (ATCC HB-8065) was obtained from the National Cancer Institute, Bangkok, Thailand. The HepG2, and A549 cells were cultured in RPMI 1640 medium (Hyclone, Logan, UT, USA). Both cell lines were supplemented with 10% heat inactivated fetal bovine serum (Hyclone), 100 U/mL penicillin and 100 µg/mL streptomycin (Gibco BRL, Grand Island, NY). All cell lines were maintained at 37°C in a 5% CO₂ incubator and the media were changed twice weekly.

In vitro cytotoxicity assay

The sulforhodamine B (SRB) assay was performed to assess growth inhibition using a colorimetric assay, which estimates cell number indirectly by staining total cellular protein with the dye SRB^[20].

Briefly, 100 µL/well of cell suspensions ($0.5-2.0 \times 10^5$ cells/mL) were seeded in 96-well microtiter plates and incubated at 37°C to allow for cell attachment. After 24 h, the cells were treated with the extract by adding 100 µL/well of each concentration in triplicate to obtain a final concentration of 0.8, 4, 12.5, 25, 50, 100, 200, 400, and 1000 µg/well for the extracts; 0.029, 0.058, 0.116, 0.174, and 0.348 µg/mL for the doxorubicin; and, 0.1, 0.2, 0.5, 1.0, 2.5, and 5.0 µg/mL for the cisplatin.

The plates were incubated for 1 h (d 0) and 72 h (d 3) at 37°C. At the end of each exposure time, the medium was removed. The cells were fixed with 20% (w/v) trichloroacetic acid (TCA, Fluka, Buchs, Switzerland) at 4°C for 1 h, stained for 30 min with 0.4% (w/v) SRB (Sigma, St. Louis, MO, USA) dissolved in 1% acetic acid (Sigma) for 30 min, and washed four times with 1% acetic acid. The protein-bound dye was solubilized with 10 mmol/L Tris base, pH 10 (Sigma).

The absorbance (OD) of each well was read on an ELISA plate reader (Amersham, Buckinghamshire, UK) at 492 nm. Percentage of cell survival was calculated using the formula: Percentage cell survival = [(OD test sample at d 3 - OD d 0)/(OD control at d 3 - OD d 0)] × 100.

Dose-response curves were plotted, and 50% growth inhibitory concentrations of extracts or drugs (IC₅₀) were calculated through computation with the CalcuSyn software program (Biosoft, Cambridge, UK). By comparing the growth inhibitory effect with normal cells,

the selectivity of the extract was expressed as the extract shown to be toxic to specific types of cancer cell lines.

Evaluation of drug interaction

Combination assays were performed using appropriate concentrations of *P. emblica* and *T. bellerica* extracts (4, 8, 16, 24, and 48 µg/mL for A549 cells, and 25, 50, 75, 100, and 200 µg/mL for HepG2 cells) with appropriate concentrations of doxorubicin (0.029, 0.058, 0.116, 0.174, and 0.348 µg/mL) or cisplatin (0.1, 0.2, 0.3, 0.4, and 0.8 µg/mL). Cells treated with the same final concentrations of the extracts or chemotherapeutic drugs alone were also examined. Cell growth inhibition was determined using the SRB assay, as previously described. In the assessment of synergism, the combination index (CI) method (i.e., additivity or antagonism) according to Chou and Talalay was used^[21].

The CIs were calculated by the Chou-Talalay equation^[21], which takes into account both the potency (D_m or IC_{50}) and shape of the dose-effect curve. The general equation for the classic isobologram ($CI = 1$) is given by: $CI = (D_1)/(D_x)_1 + (D_2)/(D_x)_2$ (A) where $(D_x)_1$ and $(D_x)_2$ in the denominators are the doses (or concentrations) of D_1 (drug #1, for example, the extract) and D_2 (drug #2, for example, the doxorubicin) alone that gives $x\%$ inhibition, whereas (D_1) and (D_2) in the numerators are the doses of D_1 and D_2 in combination that also inhibits $x\%$ (i.e., isoeffective). The $(D_x)_1$ and $(D_x)_2$ can be readily calculated from the Median-effect equation of Chou *et al.*^[22]: $D_x = D_m [f_a/(1 - f_a)]^{1/m}$ (B) where D_x is the median-effect dose obtained from the anti-log of the X-intercept of the median-effect plot, $X\text{-log}(D)$ versus, $Y = \log[f_a/(1 - f_a)]$, or $D_m = 10^{-(Y\text{-intercept})/m}$, f_a is the fraction affected by dose D (e.g., 0.5 if cell growth is inhibited by 50%) and m is the slope of the median-effect plot. From $(D_m)_1$ and $(D_x)_2$ and $D_1 + D_2$, an isobologram can be constructed based on Eq. A: as $CI < 1$ indicates synergism; $CI = 1$ indicates an additive effect; and $CI > 1$ indicates antagonism.

For conservative, mutually nonexclusive isobolograms of two agents, a third term, $(D_1)(D_2)/(D_x)_1(D_x)_2$, is added to Eq. A. For simplicity, the third term is usually omitted, and thus the mutually exclusive assumption or classic isobologram is indicated. In this study, the CI values obtained from classic (mutually exclusive) calculations are given.

The dose-reduction index (DRI) defines the extent (folds) of dose reduction possible in a combination, for a given degree of effect, compared with the dose of each drug alone: $(DRI)_1 = (D_x)_1/(D_1)$ and $(DRI)_2 = (D_x)_2/(D_2)$. The relationship between DRI and CI is, therefore, expressed as: $CI = (D_x)_1/(D_1) + (D_x)_2/(D_2) = 1/(DRI)_1 + 1/(DRI)_2$

Statistical analysis

The data were analyzed using the SPSS software version 11.0. The IC_{50} of plants against cancer cells were compared with IC_{50} of normal cells and calculated by the Student's *t*-test. Differences were considered significant at $P < 0.01$.

RESULTS

Effect of herbal extracts on cell growth

The effects of *P. emblica* and *T. bellerica* extracts on the

Table 1 IC_{50} values of individual herbal extracts in human cancer cells and normal cells

Extract	IC_{50} value (µg/mL)		
	A549 ^b	HepG2 ^b	Vero (Normal)
<i>P. emblica</i>	12.18 ± 5.83	30.47 ± 6.67	157.86 ± 14.90
<i>T. bellerica</i>	27.07 ± 2.19	82.39 ± 27.09	238.70 ± 8.45

Data are shown as mean ± SD from three separate experiments. ^b $P < 0.01$ represents the statistical significance between normal cell (Vero) and each cancer cell (A549 and HepG2).

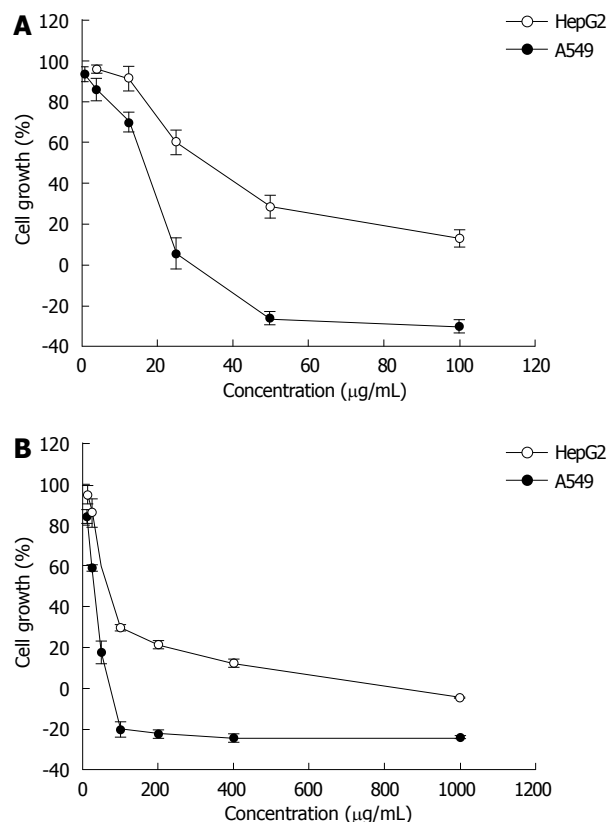


Figure 1 Cytotoxic effects of different concentrations of *P. emblica* (A) and *T. bellerica* (B) extracts on cancer cell lines. The percentage of cell growth was measured using the SRB staining assay. Each value represents the mean ± SE of three independent experiments.

proliferation of two cancer cell lines and Vero cells were determined using the SRB assay. All cell lines were growth-inhibited in a dose-dependent manner after exposure to the plant extracts (Figure 1). The IC_{50} values for *P. emblica* extract ranged from 12.18 ± 5.83 to 157.86 ± 14.90 µg/mL and for *T. bellerica* extract ranged from 27.07 ± 2.19 to 238.70 ± 8.45 µg/mL (Table 1).

When the activities of *P. emblica* and *T. bellerica* extracts against the cancer cell lines were compared with that against Vero cells and expressed as the ratio of IC_{50} values, the *P. emblica* extract had significantly different ratios of 13.0 and 5.2 against the A549 and HepG2 cells ($P < 0.01$), respectively. The *T. bellerica* extract also showed significantly different ratios of 8.8 and 2.9 against the A549 and HepG2 cells ($P < 0.01$), respectively (Table 1). It appears these two plant extracts were selectively toxic against the two cancer cell lines tested, motivating further work to determine

the combination effect of these plant extracts with chemotherapeutic drugs.

Effect of chemotherapeutic drugs on cell growth

Using the SRB assay, the effects of doxorubicin and cisplatin on the proliferation of two cancer cell lines were determined. All cell lines were growth-inhibited in a dose-dependent manner (Figure 2). The IC_{50} values for doxorubicin *vs* cisplatin ranged from 0.170 ± 0.006 to 0.511 ± 0.025 $\mu\text{g/mL}$ *vs* 1.04 ± 0.21 to 1.05 ± 0.18 $\mu\text{g/mL}$, respectively (Table 2).

Combination effects of herbal extracts with chemotherapeutic drugs on cell growth

The combination effects of *P. emblica* and *T. bellerica* extracts with doxorubicin and cisplatin in the A549 and HepG2 cell lines, as represented by the DRI, the CI and the dose-effect levels of cell growth inhibition (IC_{50} - IC_{90}), are summarized in Table 3.

The data were also examined using median effect analysis to determine the type of interactions which occurred, i.e. antagonism ($CI > 1$), additivity ($CI = 1$) or synergism ($CI < 1$) (Figures 3 and 4). With A549 cells, the combinations were synergistic at medium and high dose levels (IC_{75} and IC_{90}) for the *P. emblica*/doxorubicin and *P. emblica*/cisplatin combinations (Table 3, Figures 3 and 4). By contrast, the synergistic and additive effects were recorded at low and medium dose levels (IC_{50} and IC_{75}) for the *T. bellerica*/cisplatin combination (Table 3, Figure 4). In HepG2 cells, combinations were synergistic at low and medium dose levels (IC_{50} and IC_{75}) for the *P. emblica*/doxorubicin combination or at a low level (IC_{50}) for the *T. bellerica*/doxorubicin combination (Table 3, Figure 3). All other combinations had antagonistic effects.

The DRI showed a considerable dose reduction for herbal extracts and drugs used as a result of their synergism (Table 3). When using synergistic drug combinations at corresponding dose levels, the DRI indicated that the concentration of doxorubicin necessary to inhibit the growth of 50% of cancer cells (IC_{50}) could be decreased 1.64-fold (A549, *P. emblica*/doxorubicin) to 4.69-fold (HepG2, *P. emblica*/doxorubicin), and the IC_{90} could be reduced 2.59-fold (A549, *P. emblica*/cisplatin) to 2.60-fold (A549, *P. emblica*/doxorubicin; Table 3). The dose reduction level was different and specific to each combination and cell line.

DISCUSSION

In this study, the growth inhibitory activity of the *P. emblica* and *T. bellerica* extracts and the chemotherapeutic drugs doxorubicin and cisplatin were investigated in A549, and HepG2 cells. Our results indicate that both plant extracts and chemotherapeutic drugs mediated significant growth inhibitory effects on both cell lines tested in a dose-dependent manner (Tables 1 and 2, Figure 1 and 2).

P. emblica extract combined with doxorubicin or cisplatin resulted in synergistically enhanced growth inhibitory activity at different dose levels in A549 and HepG2 cell lines. The synergistic effects were also demonstrated when *T. bellerica* extract was combined with doxorubicin or cisplatin in HepG2 and A549 cells. The significance of this

Table 2 IC_{50} values of chemotherapeutic drugs in human cancer cells

Drug	IC_{50} value ($\mu\text{g/mL} \pm \text{SD}$)	
	A549	HepG2
Doxorubicin	0.170 ± 0.006	0.511 ± 0.025
Cisplatin	1.04 ± 0.21	1.05 ± 0.18

Data are shown as mean \pm SD from three separate experiments.

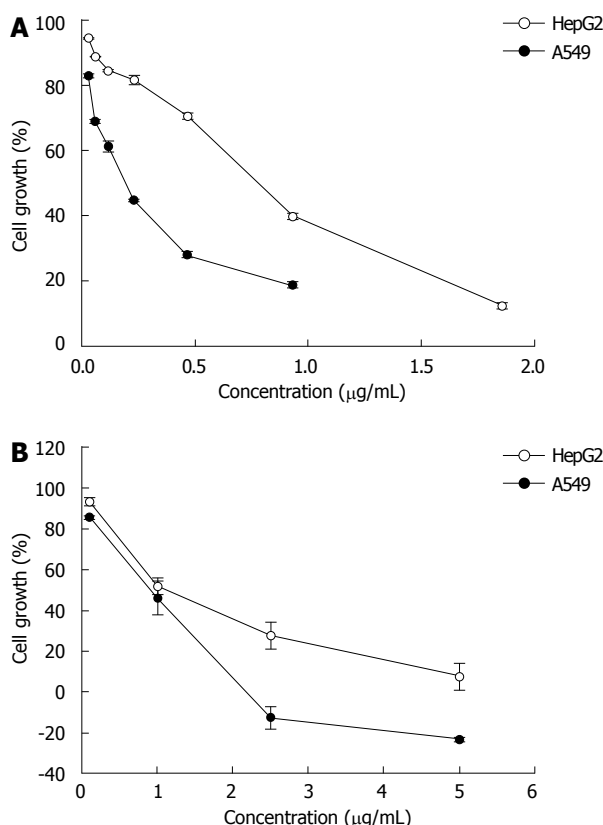


Figure 2 Cytotoxic effects of different concentrations of doxorubicin (A) and cisplatin (B) on cancer cell lines. The percentage of cell growth was measured using SRB staining assay. Each value represents the mean \pm SE of three independent experiments.

finding lies in the fact that doxorubicin and cisplatin are well-known cancer therapeutic agents, but cause high toxicity to normal tissues during cancer therapy^[23,24].

Several studies have shown that doxorubicin has harmful effects on health (i.e., immunosuppression and secondary cardiomyopathy) and can lead to the development of primary and secondary drug resistance in tumor cells thereby limiting the clinical success of cancer chemotherapy^[25,26]. Recent reports show that combination (rather than single-agent) chemotherapy is a superior modality and that naturally occurring dietary supplements with known anti-cancer activity could be used in combination chemotherapy to reduce the systemic toxicity of chemotherapeutic agents^[27,28].

Our study provides corroborative evidence as it showed that *P. emblica* and *T. bellerica* extracts were selectively toxic against two cancer cell lines and that, in combination with doxorubicin and cisplatin, produced an increased growth inhibitory effect in both A549 and HepG2 cells. Calcula-

Table 3 Dose-effect relationships of extracts and drug combinations in human cancer cell lines

Cell line	Single extracts, drugs and combinations	Parameters			CI value at			DRI value at		
		D_m ($\mu\text{g/mL}$)	m	r	IC_{50}	IC_{75}	IC_{90}	IC_{50}	IC_{75}	IC_{90}
A549	<i>P. emblica</i>	36.75	0.71	0.99				2.67	3.90	5.67
	Doxorubicin	0.164	0.78	0.99				1.64	2.07	2.60
	(D) ₁ + (D) ₂ (4:0.029)	13.75 + 0.100	0.93	0.99	0.98	0.74	0.56			
	<i>T. bellerica</i>	17.78	1.57	0.86				1.26	0.85	0.57
	Doxorubicin	0.169	0.87	0.99				1.66	1.95	2.30
	(D) ₁ + (D) ₂ (4:0.029)	14.11 + 0.102	1.00	1.00	1.40	1.69	2.18			
	<i>P. emblica</i>	18.75	0.75	0.95				1.65	2.63	4.19
	Cisplatin	0.596	0.99	0.96				2.09	2.33	2.59
	(D) ₁ + (D) ₂ (40:1)	11.38 + 0.285	1.09	0.98	1.08	0.81	0.62			
	<i>T. bellerica</i>	30.36	0.95	0.97				1.76	1.87	1.99
	Cisplatin	1.228	1.36	1.00				2.85	2.15	1.62
	(D) ₁ + (D) ₂ (40:1)	17.26 + 0.431	1.00	1.00	0.92	1.00	1.12			
HepG2	<i>P. emblica</i>	157.82	2.65	0.98				1.47	1.26	1.07
	Doxorubicin	0.584	1.04	0.96				4.69	7.58	12.23
	(D) ₁ + (D) ₂ (25:0.029)	107.23 + 0.124	1.92	0.94	0.89	0.93	1.02			
	<i>T. bellerica</i>	150.34	3.11	0.95				1.37	1.07	0.84
	Doxorubicin	0.569	1.04	0.95				4.48	7.09	11.24
	(D) ₁ + (D) ₂ (25:0.029)	109.66 + 0.127	1.83	0.97	0.95	1.07	1.28			
	<i>P. emblica</i>	111.59	2.18	0.99				1.24	1.08	0.94
	Cisplatin	0.839	0.91	0.95				2.32	4.08	7.19
	(D) ₁ + (D) ₂ (250:1)	90.395 + 0.362	1.72	0.96	1.24	1.17	1.20			
	<i>T. bellerica</i>	111.02	2.26	1.00				1.19	1.01	0.85
	Cisplatin	0.881	1.13	0.98				2.36	3.26	4.49
	(D) ₁ + (D) ₂ (250:1)	93.30 + 0.373	1.68	0.99	1.26	1.30	1.40			

Dose-effect relationships were calculated by the median-effect equation. D_m median-effect dose (concentration in $\mu\text{g/mL}$ that inhibits cell growth by 50%), m shape of the dose-effect curve (where $m = 1$, $m > 1$, and $m < 1$ indicate hyperbolic, sigmoidal, and negative sigmoidal curves, respectively), r linear correlation coefficient of the median effect plot (indicates conformity of data). CI was calculated by Chou and Talalay's CI equation. $\text{CI} < 1$, $\text{CI} = 1$, and $\text{CI} > 1$ indicate synergism, additive effect, and antagonism, respectively. D_m and m values for single drugs, extracts and their combinations were used in the equations $Dx = D_m [f_a / (1 - f_a)]^{1/m}$ and $\text{CI} = (D)_1 / (Dx)_1 + (D)_2 / (Dx)_2$. f_a fraction affected by D (e.g. 0.9 if cell growth is inhibited by 90%), $(D)_1$ and $(D)_2$ combined doses of extract 1 and drug 2 for $x\%$ inhibition, $(Dx)_1$ and $(Dx)_2$ doses of the single extract 1 and drug 2 for $x\%$ inhibition. DRI dose reduction index was measured by comparing the doses required to reach a given degree of inhibition when using the drug as a single agent and in combination.

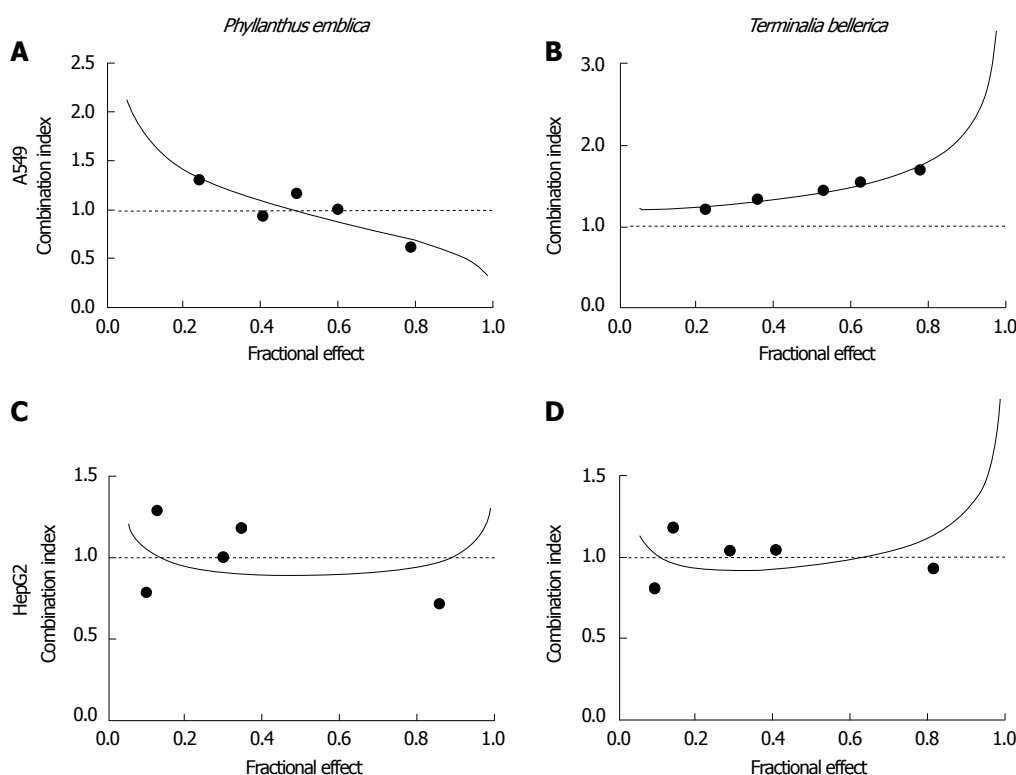


Figure 3 Combination index (CI) vs fraction affected (f_a) plots obtained from the median-effect analysis program (CalcuSyn, Biosoft, Cambridge, UK). (A) and (B) A549; (C) and (D) HepG2. Curves with solid lines are computer simulated f_a -CI plots, based on the parameters (m and D_m values) for doxorubicin and herbal extract combinations. Circles are actual combination data points. $\text{CI} < 1$, $= 1$ and > 1 indicates synergism, additive effect and antagonism, respectively.

tion of the DRI at the IC_{50} demonstrated possible reductions in doxorubicin concentrations for the drug combina-

tions ranging from 1.64-fold (*P. emblica* + doxorubicin in A549) to 4.69-fold (*P. emblica* + doxorubicin in HepG2).

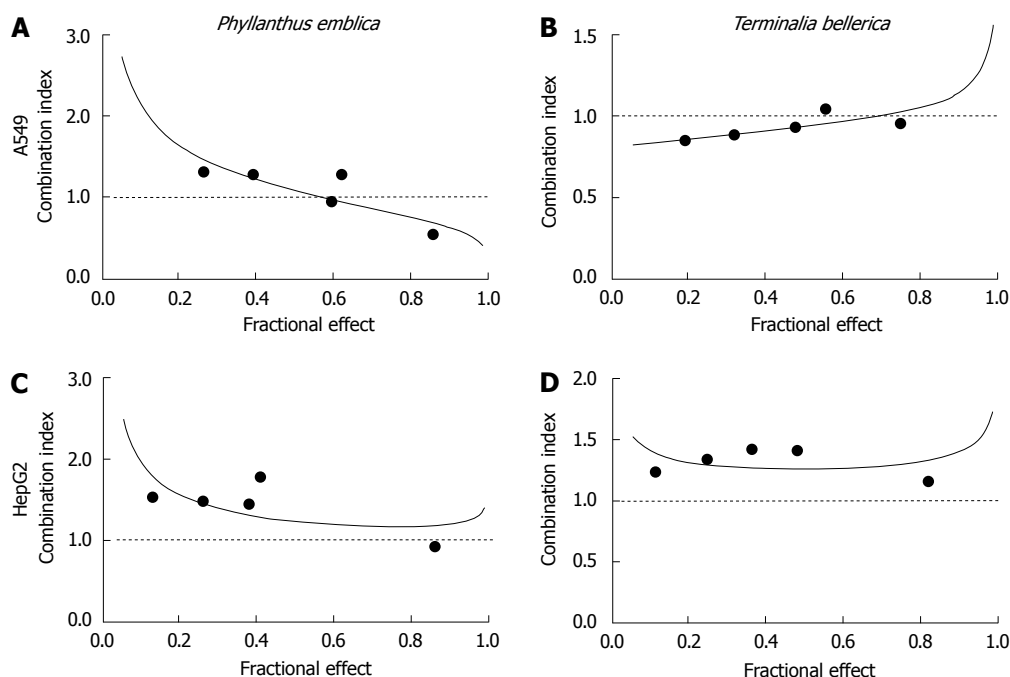


Figure 4 Combination index (CI) vs fraction affected (f_a) plots obtained from the median-effect analysis program (CalcuSyn, Biosoft, Cambridge, UK). (A) and (B) A549; (C) and (D) HepG2. Curves with solid lines are computer simulated f_a -CI plots, based on parameters (m and D_m values) for cisplatin and herbal extract combinations. Circles are actual combination data points. CI < 1, = 1 and > 1 indicates synergism, additive effect and antagonism, respectively.

This finding supports our hypothesis that combinations of plant extracts and chemotherapeutic agents allow a reduction in the dosage of the latter (i.e., doxorubicin and cisplatin), yet retaining the benefits but minimizing the cytotoxic effects, thus enhancing therapeutic efficacy.

The mechanism of action is unclear and, possibly, multiple compounds in the herbal extracts are involved^[29]. Plant derived polyphenols, including tannins and gallic acid, were reported to be the main constituents in *P. emblica* and *T. bellerica*^[13,30]. Marienfeld *et al*^[31] reported that tannic acid (TA) could inhibit the malignant cholangiocyte growth both *in vitro* and *in vivo* and also enhance sensitivity of Mz-ChA-1 cholangiocarcinoma cells to camptothecin cytotoxicity which might involve an effect on xenobiotic metabolism. In addition, TA also acts as an inhibitor of the glutathione conjugate export pump. As a result, the sensitivity of tumor cells to anticancer drugs was increased^[32,33]. This study agreed with the study of Sandhya and Mishra^[34] which showed the cytotoxic response of human breast cancer cell lines to Triphala, which contained *T. bellerica*, *P. emblica* and *T. chebula* extracts. It might be possible that *T. bellerica* and *P. emblica* extracts could induce ROS in the induction of apoptosis. Apart from the effect of tannic acid, the cytotoxic effect and apoptosis induction of gallic acid in several cancer cell lines have been reported^[13,34,35]. It was demonstrated that the effect of gallic acid on cancer cell line particularly lung cancer cells involved caspase activation and oxidative processes^[35]. In addition, gallic acid, a major component of *T. bellerica*^[14,15] has the capacity to induce apoptosis^[35] and increase the efficacy of cisplatin in combined treatment of mice transplanted with LL-2 lung cancer cells^[36]. These findings suggest that in the synergistic activity of *P. emblica* or *T. bellerica* extracts and doxorubicin it seem to be possible that the extracts could induce cytotoxic to tumor cells by a ROS mediated mechanism in cancer cells but less in normal cell^[37] as well as inhibit glutathione conjugate export pump as a result, the extracts would increase the sensitivity of A549 and HepG2 cells to doxorubicin and cisplatin.

In summary, our results demonstrate that combinations of *P. emblica* or *T. bellerica* extracts with doxorubicin or cisplatin in A549 and HepG2 cell lines have a better effect than either agent alone. Further studies are needed to assess the underlying mechanism(s), signal transduction pathways, leading to growth inhibition induced by single agents and combinations both *in vitro* and *in vivo*. A positive outcome of such studies would be increased efficacy of existing chemotherapies with reduced toxicity to the normal tissues in treatment of human lung carcinoma and hepatocellular carcinoma.

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COMMENTS

Background

Belleric myrobalan (*Terminalia bellerica*) and emblic myrobalan (*Phyllanthus emblica*), are constituents of herbal formulation (Triphala) and is widely used in folk medicine in Southeast Asia. Both extracts have been shown to inhibit cancer cell growth.

Research frontiers

Currently, a variety of effective phytochemicals have been tested in cancer treatment, which are used alone, or in combination with chemotherapeutic agents of treatment. The combinations of *P. emblica* or *T. bellerica* extracts with doxorubicin or cisplatin have a synergistic growth effect against some cancer cell lines. Depending on the combination ratio, the doses for each drug for a given degree of effect in the combination may be reduced. The mechanism of this synergistic effect is unclear and, possibly, multiple compounds in the herbal extracts are involved.

Innovations and breakthroughs

In this report, we demonstrate that combinations of *P. emblica* or *T. bellerica*

extracts with doxorubicin or cisplatin in A549 and HepG2 cell lines have a better effect than either agent alone.

Applications

P. emblica and *T. bellerica* extracts appear to be an effective against hepatocellular carcinoma and lung cancer cells and less toxic against normal cells. A combination of an effective *P. emblica* and *T. bellerica* extracts with chemotherapeutic agents which combined may be enhancing efficacy while reducing toxicity to normal tissues.

Peer review

This is a very nice experimental study about the interaction between drugs and plant extracts performed *in vitro*. In this study, the authors investigated the combination effects of *P. emblica* and *T. bellerica* extracts with conventional cytotoxic agents against human cancer cells. The paper shown a synergistic effect of *P. emblica* and *T. bellerica* extracts with doxorubicin and cisplatin against human hepatocellular carcinoma and lung cancer cells.

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H pylori

Polymorphism of -765G > C *COX-2* is a risk factor for gastric adenocarcinoma and peptic ulcer disease in addition to *H pylori* infection: A study from northern India

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association with gastric adenocarcinoma as compared to NUD [77.4% vs 29%, $P < 0.001$, odds ratio (OR) 8.20; 95% confidence interval (95% CI), 4.08-16.47] and PUD (77.4% vs 31.1%, $P < 0.001$; OR 8.04; 95% CI, 3.25-19.90). Risk of gastric adenocarcinoma was significantly higher in patients having C carrier with (OR 7.83; 95% CI 3.09-19.85) and without *H pylori* infection (OR 7.06; 95% CI, 2.61-19.09). Patients with C carrier and *H pylori* infection had significant risk for the development of PUD ($P < 0.001$; OR 5.65; 95% CI, 2.07-15.34).

CONCLUSION: -765G > C *COX-2* polymorphism with or without *H pylori* could be a marker for genetic susceptibility to gastric adenocarcinoma. *COX-2* polymorphism in presence of *H pylori* infection might be useful in predicting the risk of PUD.

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Key words: *COX-2* polymorphism; Gastric adenocarcinoma; Peptic ulcer disease; *Helicobacter pylori* infection

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Abstract

AIM: To investigate -765G > C *COX-2* polymorphism and *H pylori* infection in patients with gastric adenocarcinoma, peptic ulcer disease (PUD) and non-ulcer dyspepsia (NUD).

METHODS: We enrolled 348 adult patients (62 gastric adenocarcinoma, 45 PUD and 241 NUD) undergoing upper gastrointestinal endoscopy at two referral centers between September, 2002 and May, 2007. *H pylori* infection was diagnosed when any of the four tests (RUT, culture, histopathology and PCR) were positive. Genotyping for -765G > C polymorphism of *COX-2* was performed by PCR-RFLP analysis.

RESULTS: Frequency of C carrier had significant

INTRODUCTION

H pylori has been classified as a major cause of chronic gastritis and peptic ulcer disease (PUD), as well as a risk factor for gastric adenocarcinoma and mucosa-associated lymphoid tissue (MALT) lymphoma^[1-3]. Although there is a considerable high rate of *H pylori* infection in Asian countries such as Japan, China, Thailand, Indonesia and India, there is a remarkable difference in incidence of gastric cancer within these countries (Asian enigma)^[4,5]. The annual incidence of gastric cancer is disproportionately high in Japan and China in spite of a lower *H pylori* seropositivity^[5]. In contrast, in India

seropositivity of *H pylori* is very high, but the annual incidence of gastric cancer is low (Indian enigma)^[5]. Even studies based on differences in *H pylori* virulence factors couldn't explain such contradictory findings^[6]. This shows that *H pylori* may not be the sole factor, but may play a synergistic role with factors like diet and host genetic make up in gastric carcinogenesis^[5].

The role of host-related factors in the pathogenesis of diseases caused by *H pylori* infection has largely been ignored^[7,8]. Overexpression of cyclooxygenase-2 (COX-2) has been observed in several forms of cancer^[9-16], including gastric cancer and precancerous lesions^[17-19]. COX, (also known as prostaglandin endoperoxide synthase) is a rate-limiting enzyme for the synthesis of prostaglandins (PGs) from free arachidonic acid. Two isoforms of COX have been identified; COX-1 is constitutively expressed in most normal tissues and considered to be a housekeeping enzyme responsible for the maintenance of vascular homeostasis and gastroprotection. In contrast, COX-2 is the inducible isoform of the enzyme and is rarely expressed in normal tissues, but it is rapidly induced by bacterial lipopolysaccharide (LPS), cytokines, growth factors, mitogens and tumor promoters^[9,10]. Up-regulation of COX-2 plays an important role in the inhibition of apoptosis, tumor growth, angiogenesis, invasion and metastasis, which are considered to be important steps in cancer development^[9,17,20,21]. Several polymorphisms in COX-2 have been identified so far. However, only a few seemed to have a functional effect on the transcription. Recently, Papafili *et al*^[22] described a new polymorphism in the promoter region of COX-2, characterized by a guanine (G) to cytosine (C) transition at position -765 (-765G > C). This polymorphism appears to disrupt a stimulatory protein 1 (Sp1) binding site, which is considered to be a positive activator of transcription and leads to a 30% reduction of the COX-2 promoter activity *in vitro*^[22]. Only a few studies have been published on COX-2 polymorphisms either in cancer or related diseases^[23-29] or non-malignant diseases^[30-32]. The aim of the present study was to evaluate the role of -765G > C COX-2 polymorphism and *H pylori* infection in patients with gastric adenocarcinoma and PUD.

MATERIALS AND METHODS

Patients

We studied 348 adult patients, including 62 with gastric adenocarcinoma, 45 PUD and 241 non ulcer dyspepsia (NUD), undergoing upper gastrointestinal endoscopy at two referral centers in northern India between September, 2002 and May, 2007. The diagnosis of gastroduodenal diseases was based on clinical, endoscopic and histopathological parameters. Patients with NUD were considered as disease control in our study. The Ethics Committee of the institute granted approval for the study and consents were obtained from all the patients. Subjects who had received anti-microbial therapy, H₂ receptor blockers, proton pump inhibitors and non-steroidal anti-inflammatory drugs in the last 4 weeks before endoscopy or anti-*H pylori* treatment in the past were excluded from the study.

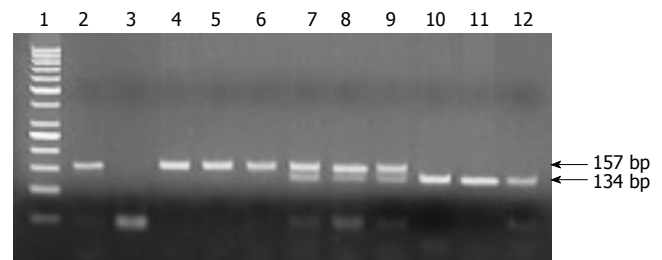


Figure 1 PCR-RFLP analysis of -765 G > C COX-2 polymorphism. Lane 1: 50 bp DNA ladder; lane 2: PCR product (undigested); lane 3: Negative control; lanes 4 to 6: Homozygous -765 CC genotype; lanes 7 to 9: Heterozygous -765 GC genotype; lanes 10 to 12: Homozygous -765 GG genotype.

DNA extraction

Genomic DNA was isolated from gastric tissues using the QIAamp DNA mini kit (QIAGEN, Hilden, Germany) as per the manufacturer's instruction.

Diagnosis of *H pylori* infection

During each endoscopy, antral biopsies were obtained and subjected to the following tests: rapid urease test (RUT), culture, histopathology and *H pylori* specific *ureA* PCR following the standard protocol as described earlier^[33]. *H pylori* infection was diagnosed if any of the above tests was positive.

-765G > C COX-2 polymorphism

Analysis of -765G > C COX-2 polymorphism was performed by PCR-based restriction fragment length polymorphism (PCR-RFLP) as previously described^[30]. The sequences of PCR primers were: forward 5'-ATTCTGGCCATCGCCGCTTC-3' and reverse 5'-CTCCTTGTTTCTTGGAAGAGACG-3' (Metabion, Martinsried, Deutschland). PCR conditions were as follows: an initial denaturation at 94°C for 10 min, followed by 35 cycles of denaturing at 94°C for 1 min, annealing at 59°C for 1 min, and extension at 72°C for 1 min. The final extension was continued at 72°C for 10 min and cooling to 4°C. Template free water was used as negative control. After PCR amplification, PCR products were subjected to restriction digestion by *Bsh* 1236I restriction endonuclease (Fermentas, Vilnius, Lithuania) for 8 h at 37°C. The DNA fragments were then separated on 3% agarose gel electrophoresis. Fragments size of 134 and 23 bp indicated the presence of a wild type homozygous -765GG genotype, a single 157 bp fragment indicated the presence of homozygous -765CC genotype and three fragments of 157, 134 and 23 bp indicated the presence of heterozygous -765GC genotype (Figure 1). The 23 bp fragment cannot be distinguished from the primer-dimer band in the agarose gel. All the experiments were repeated twice for the confirmation of RFLP results.

Statistical analysis

The data analysis was performed by SPSS software (Version 12.0, SPSS, Chicago, IL, USA). *H pylori* status in relation to gastroduodenal diseases was analyzed using Chi-square test. Multivariate logistic regression analyses were

Table 1 Demography of the study populations and *H pylori* infection

Parameter	Gastric adenocarcinoma (n = 62)	Peptic ulcer disease (n = 45)	Non-ulcer dyspepsia (n = 241)	Overall (348)
Age (yr)	56.60 ± 15.42	49.47 ± 17.22	43.75 ± 14.76	46.78 ± 15.96
Male:Female	47:15	31:14	138:103	216:132
<i>H pylori</i> infection ¹ (%)	35 (56.5)	36 (80)	133 (55.2)	204 (58.6)

¹Peptic ulcer disease vs non-ulcer dyspepsia: 80% vs 55.2%, $P = 0.002$; Gastric adenocarcinoma vs non-ulcer dyspepsia: 56.5% vs 55.2%, $P = 0.858$; Peptic ulcer disease vs gastric adenocarcinoma: 80% vs 56.5%, $P = 0.01$.

Table 2 Allelic distribution of *COX-2* polymorphism in gastric adenocarcinoma, peptic ulcer disease and non-ulcer dyspepsia

<i>COX-2</i> genotype	Gastric adenocarcinoma (n = 62)	Peptic ulcer disease (n = 45)	Non-ulcer dyspepsia (n = 241)
GG (%)	14 (22.6)	31 (68.9)	171 (71)
GC (%)	29 (46.8)	12 (26.7)	62 (25.7)
CC (%)	19 (30.6)	2 (4.4)	8 (3.3)
C carrier (%) ¹	48 (77.4)	14 (31.1)	70 (29)

¹Gastric adenocarcinoma vs non-ulcer dyspepsia: $P < 0.001$; Gastric adenocarcinoma vs peptic ulcer disease: $P < 0.001$; Peptic ulcer disease vs non-ulcer dyspepsia: $P = 0.74$.

used to identify the independent risk factors for gastric adenocarcinoma and PUD. Gender and age were included in regression analysis, and assessment for interaction was considered in the model. A two-sided P value of less than 0.05 was considered significant.

RESULTS

Patient characteristics

A total of 348 patients (mean age: 46.78 ± 15.96; 216 male) were enrolled in the study and their distributions were gastric adenocarcinoma 62 (mean age: 56.60 ± 15.42; 47 male), PUD 45 (mean age: 49.47 ± 17.22; 31 male) and NUD 241 (mean age: 43.75 ± 14.76; 138 male, Table 1).

Diagnosis of *H pylori* infection

Prevalence of *H pylori* infection in our study population was 58.6%. *H pylori* infection was significantly higher in patients with PUD than with gastric adenocarcinoma (80% vs 56.5%, $P = 0.01$) and NUD (80% vs 55.2%, $P = 0.002$, Table 1).

-765G > C *COX-2* polymorphism

All genotypic distributions were in Hardy-Weinberg equilibrium. The potential association of -765G > C *COX-2* polymorphism in patients with gastroduodenal diseases is shown in Table 2. The frequency of the -765 GG, GC and CC genotypes were 71%, 25.7% and 3.3%, in patients with NUD, 68.9%, 26.7% and 4.4% in patients with PUD and 22.6%, 46.8% and 30.6% in patients with gastric adenocarcinoma, respectively. The frequency

Table 3 *H pylori* infection and *COX-2* polymorphism as risk for gastric adenocarcinoma

<i>H pylori</i> status	<i>COX-2</i> genotype	Gastric adenocarcinoma (n = 62)	Non-ulcer dyspepsia (Controls, n = 241)	OR (95% CI)	P-value
HP-	GG	8	79	Referent	
HP-	C carriers	19	29	7.06 (2.61-19.09)	< 0.001
HP+	GG	6	92	0.83 (0.26-2.59)	0.747
HP+	C carriers	29	41	7.83 (3.09-19.85)	< 0.001

HP-: *H pylori* negative; HP+: *H pylori* positive.

Table 4 *H pylori* infection and *COX-2* polymorphism as risk for peptic ulcer disease

<i>H pylori</i> status	<i>COX-2</i> genotype	Peptic ulcer disease (n = 45)	Non-ulcer dyspepsia (Controls, n = 241)	OR (95% CI)	P-value
HP-	GG	8	79	Referent	
HP-	C carriers	1	29	0.86 (0.09-7.48)	0.89
HP+	GG	23	92	2.83 (1.18-6.73)	0.019
HP+	C carriers	13	41	5.65 (2.07-15.34)	< 0.001

HP-: *H pylori* negative; HP+: *H pylori* positive.

of C carrier was more common in patients with gastric adenocarcinoma as compared with NUD [77.4% vs 29%, $P < 0.001$, odds ratio (OR) 8.20; 95% confidence interval (95% CI), 4.08-16.47] and PUD (77.4% vs 31.1%, $P < 0.001$, OR 8.04; 95% CI, 3.25-19.90). However, the frequency of C carrier in patients with PUD and NUD was similar (PUD vs NUD 31.1% vs 29%, $P = 0.74$; OR 1.13; 95% CI, 0.56-2.27).

Interaction between *H pylori* infection and -765G > C *COX-2* polymorphism

We also examined the potential interaction between *H pylori* infection and -765G > C *COX-2* polymorphism in the development of gastric adenocarcinoma and PUD in our population. Presence of C carrier with ($P < 0.001$, OR 7.83, 95% CI, 3.09-19.85) and without *H pylori* infection ($P < 0.001$, OR 7.06, 95% CI, 2.61-19.09) was significantly associated with gastric adenocarcinoma (Table 3). We also found that patients with C carrier and *H pylori* infection had significant risk for the development of PUD ($P < 0.001$, OR 5.65, 95% CI, 2.07-15.34; Table 4).

DISCUSSION

We investigated the potential association of -765G > C *COX-2* polymorphism and *H pylori* infection with gastric adenocarcinoma and PUD. We report for the first time that -765G > C *COX-2* polymorphism with or without *H pylori* infection could be a marker for genetic susceptibility to gastric adenocarcinoma. To the best of our knowledge, this is the first study to show that patients with C carriers of *COX-2* gene are susceptible to develop PUD in the presence of *H pylori* infection.

Table 5 C allele frequency and C carrier distribution in control populations from various countries

Country	Control population (n)	C allele frequency (%)	C carrier distribution (%)	Reference
America				
USA	228	21	37	27
USA (African American)	100	32	52	27
Europe				
Portugal	210	22	38	34
Italy	864	28	50	31
UK	454	14	25	22
Poland	547	17	31	30
Australia				
Australia	168	17	31	32
Asia				
India	241	16	29	Present study
Singapore	1177	5	9	23
Japan	241	2	5	24
China	1270	2	4	19

Genetic polymorphism is considered an important determinant for the development of cancer. An association between increased COX-2 gene expression and cancer including gastric adenocarcinoma has been reported^[17-19]. A new polymorphism in the promoter region of COX-2, characterized by a guanine (G) to cytosine (C) transition at position -765 (-765G > C) appears to disrupt Sp1 binding site, which reduces the COX-2 promoter activity to the extent of 30% *in vitro*^[22]. An altered susceptibility to develop cancer due to the disruption in Sp1 binding site is attributed to this polymorphism (-765G > C) of COX-2 gene^[22,23]. The frequency of this polymorphism seems to vary, especially among different ethnic populations (Table 5). C allele and C carriers have been frequently reported in the western populations than the Asians. Both C allele (16.2%) and C carriers (29%) in our control populations are more close to the West, but much higher than the other Asian countries^[19,22-24,27,30-32].

In our study, -765 C carriers were frequently present in gastric adenocarcinoma as compared to disease controls (Table 3). Patients with C carriers had 8.2-fold increased risk of progression to gastric adenocarcinoma. Pereira *et al* reported nearly 3-fold increased risk of progression to gastric adenocarcinoma in patients with atrophy or intestinal metaplasia carrying C allele^[34]. Zhang *et al* also reported that patients with C carriers had 2.66-fold increased risk of gastric adenocarcinoma^[35]. Recently, Guo *et al* described a 2-fold increased risk of esophageal squamous cell carcinoma due to the polymorphism in COX-2 gene^[36]. It appears that the risk for the development of gastric adenocarcinoma related to COX-2 polymorphism in our population is much higher than other published studies. Although the exact molecular mechanism by which COX-2 polymorphism may increase the risk of gastric adenocarcinoma development is still unclear, studies in the COX-2 promoter revealed that COX-2 transcription is activated by E2 promoter binding factor 1 (E2F1)^[37,38]. Hence, the ability of this polymorphism to create an E2F binding site, essential for the expression of several genes may be the reason for the increased risk^[30]. The

contribution of genetic polymorphism to the risk of gastric adenocarcinoma may depend on the study population as well as on several environmental and dietary factors. Therefore, each population has to be evaluated for its own genetic profile for cancer risk that may help to understand the geographic and racial differences for development of gastric adenocarcinoma^[39]. When we analyzed the combination of C carrier and *H pylori* infection, the risk was nearly 8-fold (OR 7.83; 95% CI, 3.09-19.85) in *H pylori* positive individuals and 7-fold (OR 7.06, 95% CI, 2.61-19.09) in *H pylori* negative individuals. So far, association between *H pylori* infection and COX-2 gene polymorphism in the development of gastric adenocarcinoma has not been studied. The present study clearly shows that C carriers either in presence or absence of *H pylori* infection are susceptible to develop gastric adenocarcinoma. The frequency of C carriers was almost equal in our patients with PUD (31.1%) and disease controls (29%). But the combination of C carrier and *H pylori* infection had nearly 6-fold increased risk to develop PUD (OR 5.65, 95% CI 2.07-15.34). Interestingly, the risk was not increased in *H pylori* negative individuals, implicating a potential interplay between *H pylori* infection and COX-2 polymorphism in the development of PUD (Table 4). There are no data available in literature to compare our observations.

In conclusion, this study suggests that -765G > C COX-2 polymorphism could be a marker for genetic susceptibility to gastric adenocarcinoma. This polymorphism in gastric adenocarcinoma was independent to *H pylori* infection. However, patients with *H pylori* infection and COX-2 polymorphism had higher risk to develop PUD. Thus, COX-2 polymorphism might be useful in predicting the risk of PUD in presence of *H pylori* infection. Further studies on different ethnic groups are warranted to confirm the association of this polymorphism with the risk of gastric adenocarcinoma and PUD.

COMMENTS

Background

It remains unclear why only a subpopulation of *H pylori* infected individuals develop peptic ulcer disease (PUD) and gastric adenocarcinoma. This raises the possibility that host genetic factors play an important role in the pathogenesis of *H pylori* infection. Differential expression of COX-2 enzyme might confer inter-individual susceptibility to gastric cancer and PUD. Hence we investigated -765G > C COX-2 polymorphism and *H pylori* infection in patients with gastric adenocarcinoma, PUD and non ulcer dyspepsia (NUD).

Research frontiers

The role of host-related factors in the pathogenesis of diseases caused by *H pylori* infection has largely been ignored. Role of COX-2 polymorphism in patients with gastric adenocarcinoma and PUD in presence or absence of *H pylori* infection has not been studied. The study showed that COX-2 polymorphism was associated with gastric adenocarcinoma independent of *H pylori* infection. However, patients with combined *H pylori* infection and COX-2 polymorphism had higher risk to develop PUD. Further studies on different ethnic groups are warranted to confirm the association of this polymorphism with the risk of gastric adenocarcinoma and PUD.

Innovations and breakthroughs

We report for the first time that -765G > C COX-2 polymorphism with or without *H pylori* infection could be a potential marker for genetic susceptibility to gastric adenocarcinoma. To the best of our knowledge, this is the first study to show that patients with C carriers of COX-2 gene are susceptible to develop PUD in the presence of *H pylori* infection.

Applications

Detection of COX-2 polymorphism might help to identify the subgroup of patients having greater susceptibility to develop gastric adenocarcinoma, and peptic ulcer disease in presence of *H pylori* infection.

Peer review

COX-2 polymorphism and its role in carcinogenesis is an extremely exciting field. Most of the work in this area has been done in the investigation of colorectal cancer, more because of the relatively higher incidence of colorectal cancer and its ease of detection. This is probably the first study to document the role of COX-2 polymorphism in gastric cancer and PUD in addition to *H pylori* infection.

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BASIC RESEARCH

Apoptosis of human pancreatic cancer cells induced by Triptolide

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Abstract

AIM: To investigate apoptosis in human pancreatic cancer cells induced by Triptolide (TL), and the relationship between this apoptosis and expression of caspase-3, bcl-2 and bax.

METHODS: Human pancreatic cancer cell line SW1990 was cultured in DMEM media for this study. MTT assay was used to determine the cell growth inhibitory rate *in vitro*. Flow cytometry and TUNEL assay were used to detect the apoptosis of human pancreatic cancer cells before and after TL treatment. RT-PCR was used to detect the expression of apoptosis-associated gene caspase-3, bcl-2 and bax.

RESULTS: TL inhibited the growth of human pancreatic cancer cells in a dose- and time-dependent manner. TL induced human pancreatic cancer cells to undergo apoptosis with typically apoptotic characteristics. TUNEL assay showed that after the treatment of human pancreatic cancer cells with 40 ng/mL TL for 12 h and 24 h, the apoptotic rates of human pancreatic cancer cells increased significantly. RT-PCR demonstrated that caspase-3 and bax were significantly up-regulated in SW1990 cells treated with TL while bcl-2 mRNA was not.

CONCLUSION: TL is able to induce the apoptosis in human pancreatic cancer cells. This apoptosis may be mediated by up-regulating the expression of apoptosis-associated caspase-3 and bax gene.

INTRODUCTION

Pancreatic adenocarcinoma is characterized by a poor prognosis and lack of response to conventional therapy. The incidence has shown that no significant sign of decline throughout the past 20 years and almost equals its mortality^[1-3]. The 5-year survival rate for this disease is less than 4% and the median survival time after diagnosis is less than 6 mo^[2,3]. Surgical resection of the tumor is still the only effective treatment option, although only 20% of carcinomas of the head of the pancreas are resectable^[4]. Furthermore, the median survival even after apparent curative resection is only 20 mo, because of early tumor recurrence or rapid metastatic spread^[2,4]. Other treatment options, such as chemotherapy or radiation therapy, provide limited palliation without significant improvement of survival in patients with unresectable pancreatic cancer^[2]. Therefore, new targets for chemo-preventive and therapeutic agents need to be identified.

Triptolide (TL), extracts of the Chinese herb *Tripterygium Wilfordii* Hook have potent anti-inflammatory and immunosuppressive properties and have been used successfully in traditional Chinese medicine for the treatment of rheumatoid arthritis and lupus erythematosus^[5,6]. It has been recently reported that TL possesses anti-tumor and proapoptotic activities in many different tumor cell lines, including breast, prostate, lung, and leukemia cells lines^[7-13]. TL was also shown to

sensitize cells to death induced by a variety of agents, such as Apo2/Trail, TNF- α , and different chemotherapeutic agents^[14-16]. In this study, we provide evidence that TL potently inhibits human pancreatic cancer cell lines growth *in vitro*, suggesting that TL could be used to prevent or treat pancreatic cancer in the future. Considerable studies indicated that TL functioned through a p53-dependent or independent way^[11,14,17,18]. Recently, Bing *et al* suggested that TL induced caspase-dependent cell death via the mitochondrial pathway in leukemia cells^[19]. However, the cellular and molecular mechanisms underlying TL-induced apoptosis in tumor cells are not fully understood.

The purpose of this study is to investigate the inhibitory effects of TL on apoptosis and angiogenesis of pancreatic cancer *in vitro* and further to explore whether TL exerts clinical therapeutic value for patients with pancreatic cancer.

MATERIALS AND METHODS

Cell culture

The human pancreatic cancer cell line SW1990 was obtained from Professor Wang Xing Peng, Shanghai JiaoTong University (Shanghai, China). SW1990 was cultured in DMEM media. Media was supplemented with 10% FBS and cells grown as monolayers in a humidified atmosphere at 37°C. Crystalline TL (PG490, purity 99%) was obtained from the Institute of Dermatology, Chinese Academy of Medical Sciences (Nanjing, PR China), and prepared as previously described^[18].

MTT assay

Cells were inoculated onto 96-well plates at the density of 4×10^3 cells/well for 24 h, then treated with various concentrations of TL (100 μ L/well), and incubated for 6, 12, 24 and 48 h, respectively. Then, 5 g/L MTT solution (20 μ L/well) was added to each well, and cells were incubated for an additional 4 h at 37°C. The supernatant was aspirated, and 100 μ L of DMSO was added to the wells to dissolve any precipitate present. The suspension was placed on micro-vibrator for 5 min and the absorbance (A) was then measured at 570 nm by an enzyme immunoassay instrument. Cell inhibitory ratio was calculated by the following formula: Inhibitory ratio (%) = $(1 - A_{570} \text{ of treated group}) / (A_{570} \text{ of control group}) \times 100\%$. IC50 was calculated by SAS statistical software.

Flow cytometry

An early indicator of apoptosis is the rapid translocation and accumulation of the membrane phospholipid phosphatidylserine from the cytoplasmic interface to the extracellular surface. This loss of membrane asymmetry can be detected using the binding properties of Annexin V. To detect apoptosis, Annexin V binding assay was used. After treatment TL 40 ng/mL for 24 h, cells were collected. Cell suspension was added with 10 μ L of fluorescein-conjugated Annexin V (10 μ g/mL) and 10 μ L of PI reagent (50 μ g/mL) and detected by flow cytometry.

TUNEL assay

Cells were seeded and set up in chamber slides (Becton Dickinson, Franklin Lakes, NJ), then analyzed for in site apoptosis using the terminal deoxynucleotidyl transferase (TdT)-mediated d-UTP nick end labeling (TUNEL) method. The DNA fragments in apoptotic cells were labeled at free 3'-OH DNA ends and DNA strand breaks. Incorporated fluorescein was detected by anti-fluorescein antibody conjugated with alkaline phosphatase. After substrate reaction stained cells were analyzed with light microscopy.

Real-time PCR

The human pancreatic cancer cells were treated in the presence or absence of 40 ng/mL, 80 ng/mL, 160 ng/mL TL for 48 h and total RNA was extracted with TRIzol reagent (Invitrogen Life Technologies). Real-time quantitative PCR was performed by using a cycler (Rocha) and SYBR Green Dye. The primer sequences for genes bcl-2: sense 5'-TCCATGTCCTTTGGACAACCA-3', antisense 5'-CTCCACCAGTGTTCCCATCT-3'; bax: sense 5'-TCCATGTCCTTTGGACAACCA-3', antisense 5'-CTCCACCAGTGTTCCCATCT-3'; caspase-3: sense 5'-AACTGGACTGTGGCATTG-3', antisense 5'-ACCAGGTGCTGTGGAGTA-3'; β -actin: sense 5'-AAGTACTCCGTGTGGATCGG-3', antisense 5'-ATGCATTCACCTCCCCTGTG-3'. The experimental procedure was carried out according to the QIAGEN one step RT-PCR Kit. The reaction parameters of bcl-2, bax and caspase-3 were as follows: 94°C 5 min, 94°C 40 s, 56°C 55 s, 72°C 1 min for 36 cycles, 72°C extension 7 min; 94°C 5 min, 94°C 40 s, 58°C 55 s, 72°C 1 min for 36 cycles, 72°C extension 7 min; 94°C 5 min, 94°C 40 s, 50°C 55 s, 72°C 1 min for 36 cycles, 72°C extension 7 min. Ten μ L PCR product was placed onto 1.5% agarose gel and observed by EB staining using Gel-Pro analyzer.

Statistical analyses

All data were expressed as mean \pm SD. Intergroup comparisons were made using one-way analysis of variance (ANOVA). A *P* value less than 0.05 was considered statistically significant.

RESULTS

Effect of TL on the proliferation of SW1990 cells

The human pancreatic cancer cells were exposed to increasing concentrations (20 ng/mL to 160 ng/mL) of TL for 6 h to 48 h, respectively. The human pancreatic cancer cells showed death in a dose- and time-dependent manner. The data are summarized in Figure 1.

TL induced apoptosis in SW1990 cells

Although previous studies reported that TL treatment showed anti-proliferation and induction of apoptosis in some cancer cells, there is heretofore no data on the usage of TL in pancreatic cancer. In the present study, we tested the effects of TL on the viability of the human pancreatic cancer cell line SW1990. Our results showed that treatment with TL resulted in a dose-dependent decrease in viable

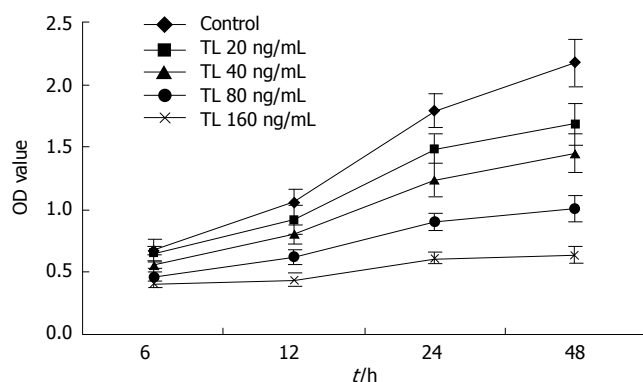


Figure 1 The effect of TL on the growth of SW1990 cells. SW1990 cells were treated with various concentrations of TL for 6 h, 12 h, 24 h or 48 h and viability was determined by MTT assay.

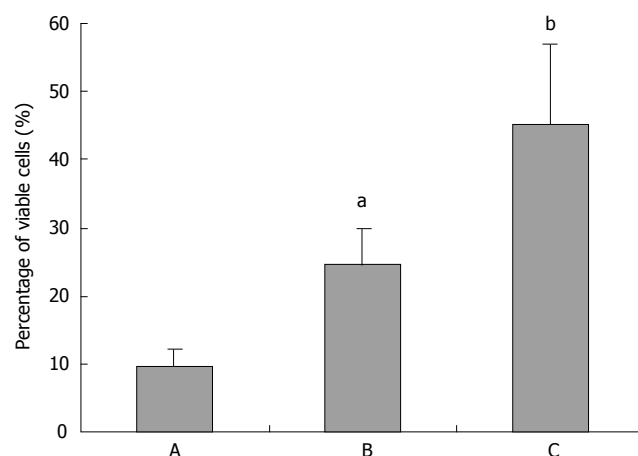


Figure 2 Triptolide induces significant cell death in pancreatic cancer cell line SW1990. A: TL 0 ng/mL; B: TL 40 ng/mL; C: TL 160 ng/mL. ^a $P < 0.05$, ^b $P < 0.001$.

cells in SW1990 cells (Figure 2). After treatment with 160 ng/mL TL for 24 h, the percentage of dead cells was 45.1% in TL cells, while that was only 9.6% in the control group.

We examined whether the reduced cell viability by TL in pancreatic cancer cell lines was due to induction of apoptosis, because SW1990 cell line seemed to be more sensitive to TL treatment, we chose that cell line and analyzed apoptosis using Annexin V/propidium iodine staining. FCS analysis revealed typical apoptotic phenotype in cells treated with 40 ng/mL TL for 24 h (Figure 3), in contrast, control cells without TL treatment displayed a small quantity of apoptosis. Moreover, we performed TUNEL assay and demonstrated that 40 ng/mL TL treatment could significantly induce apoptosis (Figure 4). These data provided first evidence that TL can be used to inhibit human pancreatic cell line growth *in vitro* and its effect is associated with induction apoptosis.

Regulation of caspase-3, bax and bcl-2 mRNA expression by TL

After exposure to 40 ng/mL, 80 ng/mL, 160 ng/mL TL for 48 h, caspase-3 and bax mRNA expression were up-regulated remarkably in TL-treated cells. In contrast, the expression of bcl-2 mRNA was not affected by TL (Figure 5).

DISCUSSION

Recent studies revealed many properties of TL relevant to anticancer activity, and anti-inflammatory activity besides. The proliferative and proapoptotic activity of TL has been shown with many different types of cancer cells *in vitro* and *in vivo* [8,21-27]. We therefore decided to test the effects of Triptolide on primary cultures of pancreatic cancer cells to see if this compound would show antitumor activity. In this study, we found that human pancreatic cancer cells treated with TL showed death in a dose- and time-dependent manner in the MTT assay. This suggested TL was able to restrain the growth of human pancreatic cancer cells. It was found that TL has the potential to induce the apoptosis of human pancreatic cancer cells.

TL has potent anticancer activity. However, the mechanism by which TL exerts its anticancer activities

remains unclear. To explore the molecular mechanisms involved in the anticancer activity of TL, we have examined the effect of TL on the growth of pancreatic carcinoma SW1990 cells. After exposure to 40 ng/mL, 80 ng/mL, 160 ng/mL TL for 48 h, treatment of SW1990 cells with TL caused a marked up-regulation of caspase-3 and bax mRNA expression. In contrast, the expression of bcl-2 mRNA was not affected by TL. Thus it is likely that TL-induced apoptosis of pancreatic carcinoma SW1990 cells has no relation with bcl-2. The study by Wang *et al* [28] has shown that treatment of pancreatic cancer cells with gypenosides extracted from TL does not affect the Bcl-2 expression. This is the reason for our focusing on Bax proteins in exploring the effects of TL on mitochondria-dependent cell death pathway. It is shown that the expression of the Bax in SW1990 cells can be up-regulated by TL in our study. Meanwhile the expression of Bcl-2 was not affected by TL treatment.

Our previous study clearly indicated that TL was able to inhibit 5-LOX gene expression in pancreatic cell lines, which contributes to TL proapoptotic activity [29]. Wang *et al* [28] examined the effect of TL on the growth of pancreatic carcinoma PANC-1 and cervical adenocarcinoma HeLa cells. They found that TL potently suppressed cell growth and induced apoptosis in HeLa and PANC-1 cells, which was indicated by nuclear fragmentation and blebbing. In both HeLa and PANC-1 cells, apoptosis induced by TL was associated with activation of caspase-3 and caspase-8, and with cleavage of poly (ADP-ribose) polymerase and Bid. Moreover, in HeLa cells, caspase-9 is also significantly activated in response to TL. Interestingly, substitution of the 14-OH of TL with an acetyl group abrogated both its anticancer and its antiinflammatory activities. Wang *et al* [26] studies suggest that TL may exert its anticancer effects by initiating apoptosis through both death-receptor- and mitochondria-mediated pathways. Their results indicate that both the apoptosis-promoting and the antiinflammatory activities of TL depend on the 14-OH group. Liu *et al* have shown that caspase 3 is responsible for TL-induced Dendritic cell apoptosis, supported by observations that TL can

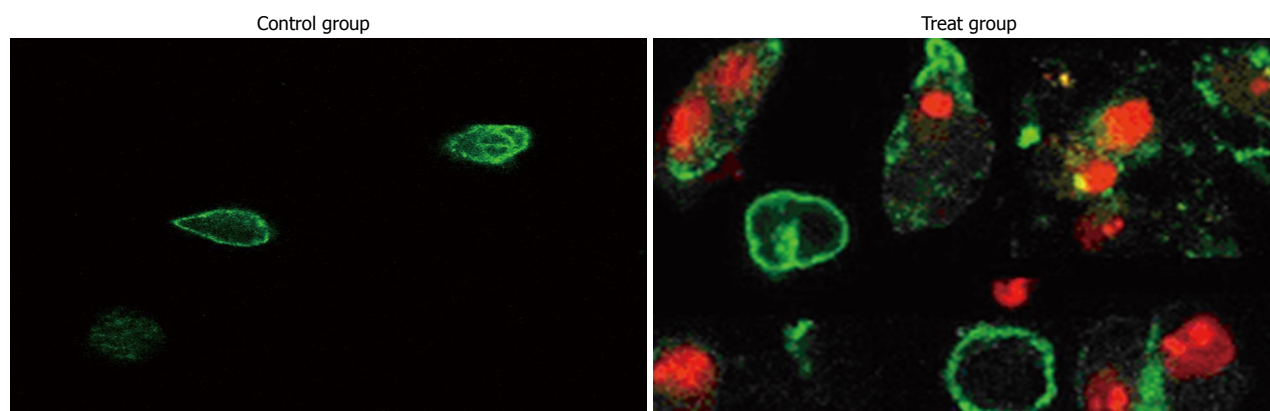


Figure 3 TL induced apoptosis in SW1990 cell lines. SW1990 cells were suspended in 100 μ L binding buffer and Annexin V/PI double staining was performed, results showed that 40 ng/mL TL induced increased number of apoptotic cells (Annexin V⁺/PI⁺).

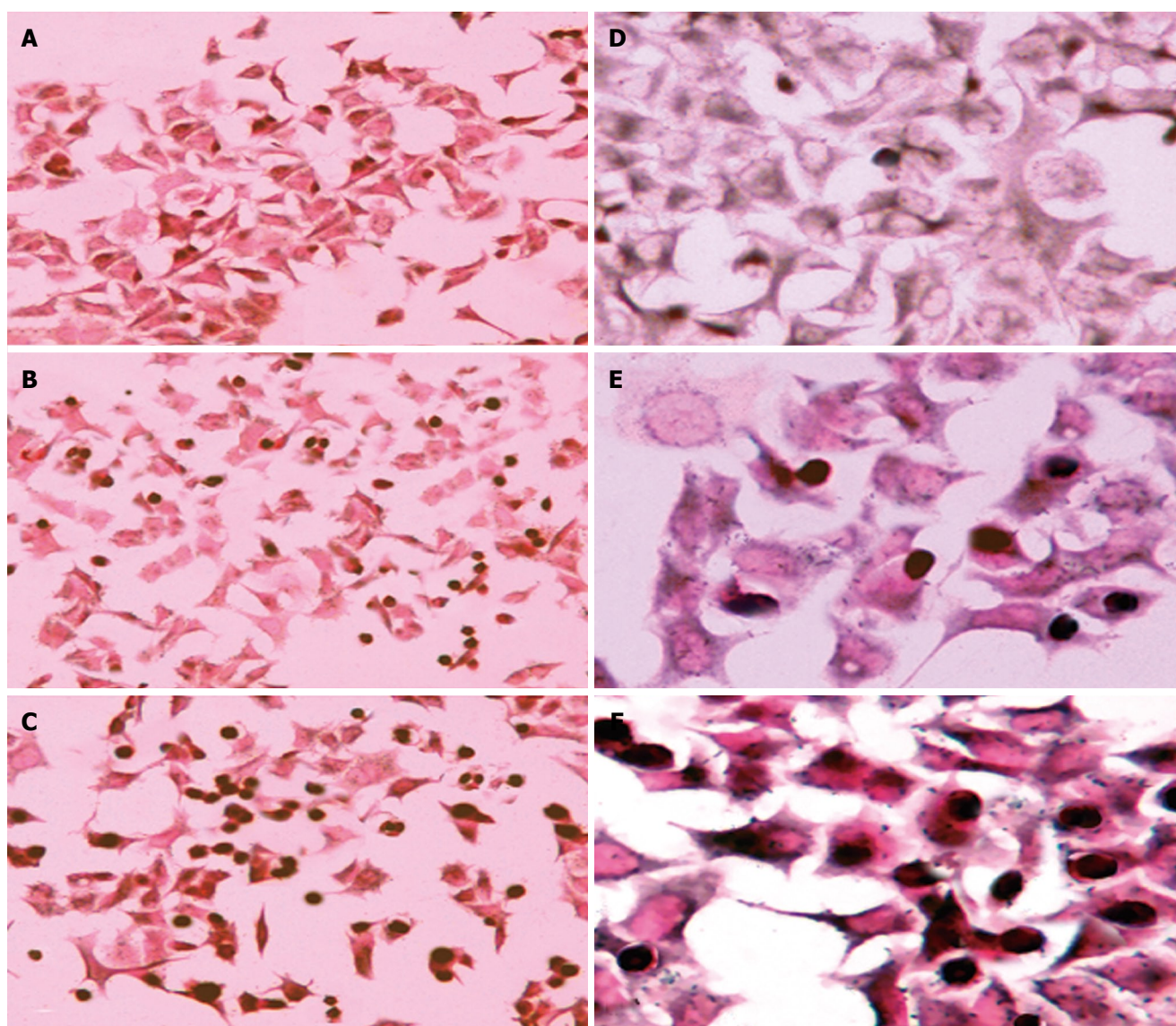


Figure 4 Apoptotic cell death was revealed by TUNEL staining after 24 h (A, B, C magnification, $\times 200$; D, E, F magnification, $\times 400$) of treatment TL. A, D (TL 0 ng/mL); B, E (TL 40 ng/mL); C, F (TL 160 ng/mL); Apoptotic cells appeared dark after staining.

activate caspase 3 and that specific inhibition of caspase 3 can abrogate the apoptotic effects of TL on Dendritic cell^[28].

The present study demonstrated that TL was able to induce the apoptosis in Human pancreatic cancer. This apoptosis may be mediated by up-regulating the

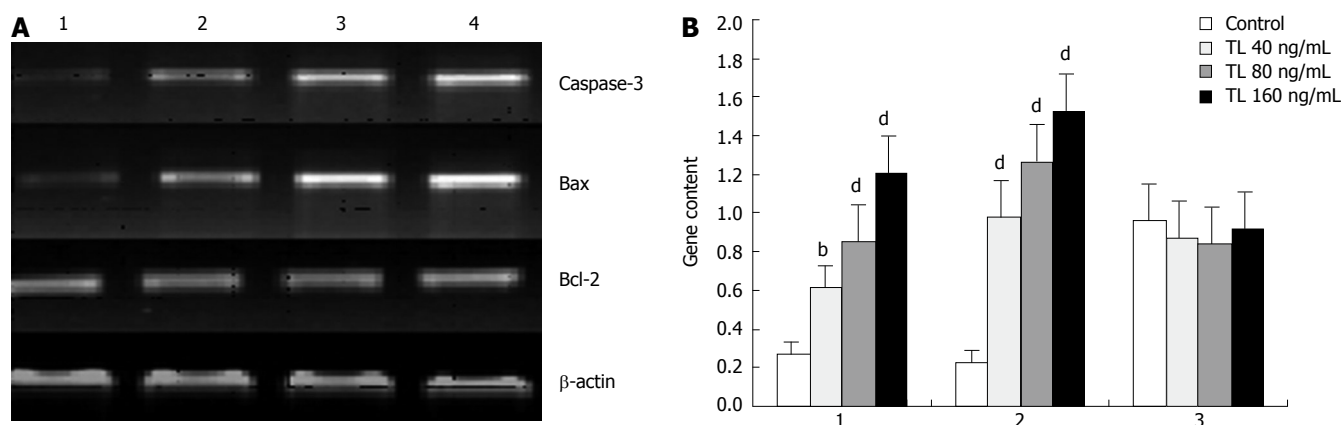


Figure 5 Detection of Bcl-2, Bax and caspase-3 gene in TL-treated SW1990 cells. Cells were treated with various concentrations of TL and Bcl-2. Bax and caspase-3 genes were analyzed by RT-PCR. **A:** 1: TL 0 ng/mL; 2: TL 40 ng/mL; 3: TL 80 ng/mL; 4: TL 160 ng/mL; **B:** 1: Caspase-3; 2: Bax; 3: Bcl-2. ^b*P* < 0.01, ^d*P* < 0.001.

expression of apoptosis-associated gene caspase-3 and bax. TL may be used as a potential anti-pancreatic carcinoma chemotherapeutic drug.

COMMENTS

Background

Pancreatic adenocarcinoma is of a poor prognosis and lack of response to conventional therapy. Among the treatment options, surgical resection of the tumor is still the most effective. The 5-year survival rate for this disease is very low even after curative resection. Therefore, new targets for chemopreventive and therapeutic agents need to be identified. Triptolide (TL) is an extract of the Chinese herb *Tripterygium Wilfordii* hook. It has been reported that TL possesses anti-tumor and proapoptotic activities in many different tumor cell lines, which functions through p53-dependent or independently^[10,12,15,16]. Recently, Bing suggested that TL induced caspase-dependent apoptosis via the mitochondrial pathway in leukemia cells. However, the cellular and molecular mechanisms underlying TL-induced apoptosis in tumor cells have not been fully understood.

Research frontiers

Pancreatic adenocarcinoma remains one of the most lethal of malignancies. The incidence of pancreatic cancer has steadily increased over the past four decades. Satisfactory treatment is available only for the minority of patients who present with very early-stage disease. There are several articles that TL possesses anti-tumor and proapoptotic activities in many different tumor cell lines, including breast, prostate, lung, and leukemia cells line. TL was also shown to sensitize cells to death induced by a variety of agents, such as Apo2/Trail, TNF- α , and different chemotherapeutic agents

Innovation and breakthroughs

This study gives us new knowledge of TL and proof that TL was able to induce apoptosis in human pancreatic cancer. This apoptosis may be mediated by up-regulating the expression of apoptosis-associated gene caspase-3 and bax. TL may be used as a potential anti-pancreatic carcinoma chemotherapeutic drug and as a clinical application in the future.

Application

The results from this study confirm that TL potently induces the apoptosis of human pancreatic cancer cells, which may be mediated by up-regulating the expression of apoptosis-associated gene caspase-3 and bax. It can be seen from this article that TL may be used to prevent or treat pancreatic cancer in the future.

Terminology

TL extracts of the Chinese herb *Tripterygium Wilfordii* hook have potent anti-inflammatory and immunosuppressive properties and have been used successfully in traditional Chinese medicine for the treatment of rheumatoid arthritis and lupus erythematosus.

Peer review

This article is of considerable interest and potential importance in demonstrating *in vitro* activity of the herbal extract TL against a human pancreatic cell line by inducing apoptosis. This work should stimulate additional *in vitro* studies in human cell lines, experiments involving animal models of pancreatic cancer, and, possibly, clinical testing of TL.

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CLINICAL RESEARCH

Hepatitis B virus genotypes in southwest Iran: Molecular, serological and clinical outcomes

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Abstract

AIM: To investigate the associations of hepatitis B virus (HBV) genotype with HBeAg and anti-HBe status, alanine aminotransferase (ALT) levels and HBV-DNA detection in different groups of HBV-infected patients in southwest Iran.

METHODS: A total of 89 HBsAg-positive serum samples were collected from the same number of patients. All sera were then investigated to determine HBV DNA and serological markers. For all the polymerase chain reaction (PCR)-positive samples, biochemical, histopathological assays and genotyping were also performed.

RESULTS: Genotype D was the only type of HBV found

in different clinical forms of acute and chronic infections. There was a high prevalence of HBeAg-negative HBV-infected patients with chronic hepatitis (52.7%). Out of 55 patients with chronic hepatitis, seven (12.7%) were diagnosed with cirrhosis. A significant association between the presence of anti-HBe antibody and an increase in ALT level, among either HBeAg-negative ($P = 0.01$) or HBeAg-positive ($P = 0.026$) patients, was demonstrated. No significant differences were observed between the clinical outcomes of HBeAg-positive and -negative individuals ($P = 0.24$).

CONCLUSION: Genotype D has been recognized as the only type of HBV found in different clinical forms of HBV infections, including cirrhosis, among the residents of southwest Iran. Anti-HBe possibly plays a role in disease progression in some patients with chronic hepatitis, at least for a period of disease.

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Key words: Hepatitis B virus-D; Cirrhosis; Iran; Anti-HBe; Polymerase chain reaction

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INTRODUCTION

Infection with hepatitis B virus (HBV) is associated with a broad range of clinical infections, including acute hepatitis, asymptomatic carrier, chronic hepatitis, cirrhosis and hepatocellular carcinoma. The course of HBV infection depends on many factors, such as host immune status, age at infection, level of viral replication and probably the

genetic variability of the virus influencing the expression of viral antigens^[1]. Heterogeneity in the global distribution of HBV genotypes may account for differences in the clinical outcomes of patients infected with HBV, and their different responses to antiviral treatment^[2,3].

DNA sequencing of HBV isolates has revealed the existence of 8 viral genotypes A-H, which vary in geographic distribution^[4]. Genotypes B and C are dominant in the Far East and south-east Asia where HBV infection is highly endemic^[5,6]. Some studies have found more severe liver disease to be associated with genotype C, as compared with genotype B^[7]. Genotype C and reactivation of Hepatitis B is associated with increased risk of cirrhosis. By contrast, genotypes A and D are more common in Western Europe and North America^[8,9]. A large cross-sectional study in Sweden showed that genotype D is associated with more active disease than genotype A^[10]. Genotype D is also predominant in the Mediterranean area as well as in the Middle East, including India. It has been associated with anti-HBe-positive chronic Hepatitis B infection in the Mediterranean region^[10,11]. However, the clinical relevance of HBV genotypes isolated from different geographical regions is poorly understood.

Based on HBsAg detection, Iran is located in an intermediate endemic region for chronic HBV infection in the Middle East, and patients with chronic HBV infection are presented with different clinical pictures. The first report of HBV genotyping of 26 HBV isolates from Iranian chronically HBV-infected individuals revealed that HBV genotype D is dominant in Iran^[12]. Nevertheless, the clinical and serological statuses of patients infected with HBV-D in this geographic region need to be further investigated.

Our aim in this study was to investigate: (1) The prevalence of different HBV genotypes in HBsAg-positive individuals living in southwest Iran, and (2) the association of HBV genotype with HBeAg and anti-HBe status, ALT levels and HBV-DNA detection in different groups of HBV-infected patients.

MATERIALS AND METHODS

Subjects

A total of 89 HBsAg-positive serum samples were collected from the same number of Iranian patients (68 males and 21 females, aged between 15 and 77 years) attending the Gastroenterology and Hepatology Clinic at the Department of Internal Medicine, Shiraz University of Medical Sciences, Shiraz, southwest Iran. Patients were registered irrespective of HBeAg status, ALT level, HBV DNA level or antiviral treatment status. Patients were excluded for hepatitis C virus (HCV), hepatitis D virus (HDV) or HIV co-infection. All patients were then tested for the following: HBeAg, antibodies to HBeAg (anti-HBe), anti-HBc, HBV DNA by polymerase chain reaction (PCR) assay, liver panel [aspartate aminotransferase (AST), ALT upper limit of normal (ULN), 667 nkat/L], alkaline phosphatase (ALP), albumin and total bilirubin], complete blood count, international normalized ratio (INR), and α -fetoprotein (AFP). An abdominal ultrasound was

also performed to determine if there were features of cirrhosis. Liver biopsy was performed based on clinical indications. Liver damage was graded (0-8) according to the inflammatory components and staging (fibrosis; 0-6) was investigated using the modified histological activity indexing^[13]. The definitions and diagnostic criteria for clinical terms were adopted from American Association for the Study of Liver Disease (AASLD) practice guidelines^[14].

Serological markers

All serological tests were performed as instructed by the manufacturers. HBsAg, anti-HBc-IgG and IgM antibodies, anti-HDV antibody, HBeAg and anti-HBe antibody were measured using commercially available standard one-step enzyme immunoassay kits (MonoLISA, Bio-Rad, France). Anti-HCV (third generation assay) was measured by enzyme immunoassay (EIA) according to the manufacturer's instructions (Innogenetics, Belgium).

PCR assay

Preparation of DNA samples from the sera: Strict measures were adopted to prevent any contamination. Purification of DNA samples was performed using a previously described method^[15].

PCR amplification and detection of HBV genotypes:

DNA amplification and detection of HBV genotypes were performed based on a nested PCR assay, using the type-specific primers previously described^[16]. Six genotypes (A-F) of HBV were identified by the assay system. To test the validity of the results, detection of HBV genotypes was also performed by quantitative real time PCR using a previously described method^[12]. Quantitative real time PCR assay was carried out using SYBER-Green signal detection. A standard curve was constructed using ten-fold serial dilutions (10^6 - 10^{11} copies/L) of plasmid DNA including the complete clinically isolated HBV-genome.

Detection of PCR product: Ten milliliters of reaction product was electrophoresed in a 1.5% agarose gel made in Tris-acetated-EDTA (TAE) buffer, pH = 8-8.5, and visualized by UV illumination after ethidium bromide (10 mg/L) staining.

Statistical analysis

Fisher's exact test, chi-square test with Yate's correction and the Student's *t* test were used where appropriate. $P < 0.05$ was considered statistically significant.

RESULTS

All 89 patients were found to be infected with HBV of genotype D. Genotype D was the only detected type found in different clinical forms of acute and chronic infections, in all HBeAg-positive and -negative patients, in all patients who had elevated or normal ALT levels and at all ages. Thirty-two (36%) out of the 89 patients were categorized as inactive HBsAg carriers. Two patients (2.2%) were diagnosed to have acute hepatitis. The remaining 55 (61.8%) were classified as having chronic hepatitis. Based on histological, clinical and laboratory findings, seven

(12.7%) patients out of 55 were diagnosed as having cirrhosis. The cirrhotic patients consisted of 6 males (85.7%), aged 23 to 77 (average; 49.6 years) and one female (14.3%) aged 52 years. All cirrhotic patients had ALT levels that were at the upper limit for the normal level, but the ALT level was lower than the AST level in all patients. Four (57.1%) of the 7 cirrhotic patients were HBeAg negative. Based on HBeAg serology results, the 55 patients with chronic hepatitis were subdivided into two groups: (1) 26 patients (47.3%) positive for HBeAg, and (2) 29 patients (52.7%) negative for HBeAg. In the latter group, 24 (82.8%) patients had an ALT level that was higher than the normal value. Twenty-three of these were positive for anti-HBe antibody, indicating a possible genetic mutation in the precore/core region of the HBV-DNA genome. However, no significant correlation between the presence or absence of HBeAg and an increase in the level of ALT was observed in patients with chronic hepatitis ($P = 0.13$).

Thirty-two patients with chronic hepatitis (58.2%) were positive for anti-HBe. Significant associations between the presence of anti-HBe antibody and an increased ALT level among both HBeAg negative ($P = 0.01$) and HBeAg positive ($P = 0.026$) individuals were observed. The number of individuals who had HBV DNA levels $> 10^9$ /L was higher among HBeAg-positive patients (11/26) than among HBeAg-negative subjects (4/29) ($P = 0.01$).

None of the 32 inactive HBsAg carriers demonstrated ALT levels higher than the normal value. They all were negative for HBeAg, but positive for anti-HBe antibody.

Based on histopathological status, more damage to hepatocytes was demonstrated in patients with chronic active hepatitis who were positive for anti-HBe compared with patients who were anti-HBe negative ($P = 0.001$). The laboratory results for the patients are presented in Table 1.

DISCUSSION

HBV has eight genotypes, which have distinct geographical distributions. There is some evidence the long-term prognosis and the initial clinical picture and response to treatment may differ depending on the genotype of the HBV having infected the patient^[17]. The viral genome controls antigen expression, leading to different genotypes and a disease spectrum after infection. Genotype D is dominant in the Mediterranean region^[18], the Middle East^[19] and Central Asia^[20]. However, the clinical outcomes of the individuals infected with HBV of genotype D are still controversial. HBV genotype D is reported to be related to acute self-limited hepatitis^[9]. Furthermore, HBV genotype D has been found in the majority of asymptomatic carriers (84.2%) and it is not found in patients with liver cirrhosis and hepatocellular carcinoma^[20]. These findings are in contrast with other studies^[22,23]. No association between HBV of genotype D and distinct clinical phenotypes has been found in the Turkish population infected with HBV^[18].

In our study, HBV-D was the only detectable genotype in different clinical forms of HBV infections, in patients with acute (2.2%), inactive HBsAg (36%) or chronic hepatitis (61.8%). Seven (12.7%) out of 55 patients with chronic hepatitis were diagnosed with cirrhosis. Genotype

Table 1 Laboratory results for patients infected with hepatitis B, virus genotype D n (%)

Test	CH HBeAg ⁺ ($n = 26$)	CH HBeAg ⁻ ($n = 29$)	Inactive HBsAg carriers ($n = 32$)	Cirrhosis ($n = 7$)
Anti-HBe ⁺	16 (61.5)	23 (79.3)	30 (93.8)	3 (43.0)
Anti-HBe ⁻	10 (38.5)	6 (20.7)	2 (6.2)	4 (57.0)
ALT > 667 nkat/L	16 (61.5)	20 (69.0)	0 (0)	7 (100.0)
ALT < 667 nkat/L	10 (38.5)	9 (31.0)	32 (100.0)	0 (0)
DNA molecules $< 10^6$ /L	2 (7.7)	2 (6.9)	17 (53.1)	3 (43.0)
DNA molecules 10^6 - 10^7 /L	7 (26.9)	14 (48.3)	13 (40.65)	1 (14.2)
DNA molecules 10^8 - 10^9 /L	6 (23.1)	9 (31.0)	2 (6.25)	2 (28.6)
DNA molecules $> 10^9$ /L	11 (42.3)	4 (13.8)	0 (0)	1 (14.2)

CH: Chronic hepatitis.

D was also found in three patients with a definite diagnosis of hepatocellular carcinoma. Nevertheless, these patients were excluded from the study, because their complete clinical and serological data were not available.

The appearance of anti-HBe usually marks non-replicative viral infection and inactive disease or response to treatment. However, 58.2% of the samples collected from patients with chronic hepatitis containing genotype D were anti-HBe positive. It should be noted that, in Iran, about 58% of HBV-infected individuals are infected with precore mutants, and may have anti-HBe antibodies in spite of actively replicating the virus^[24]. The findings of this study confirm the presence of a significant association between the presence of anti-HBe antibodies and increased ALT levels among both HBeAg-negative ($P = 0.01$) and HBeAg-positive ($P = 0.026$) individuals. These results suggest that anti-HBe may play a role in the progression of the disease.

Apart from HBeAg status, 65.5% of our patients with chronic hepatitis showed ALT levels that were higher than the normal value. In contrast to a study by Yalcin *et al*^[18], diagnosis of cirrhosis, as well as hepatocellular carcinoma, among our patients with chronic hepatitis indicated that this may be associated with ethnic background. Ethnic background might also be an influencing factor on disease progression in patients infected with HBV-D.

Although the number of HBeAg-positive patients with HBV DNA levels higher than 10^8 molecules/L was less than that of HBe-negative individuals (23% *vs* 31%), no statistically significant differences were demonstrated between the clinical outcomes of the two groups ($P = 0.24$).

In conclusion, this study suggests the unique characteristic of HBV-D infection is related to geographical location as well as ethnicity. Based on ALT levels and histopathological outcomes, we assume that anti-HBe plays a role in disease progression in some patients with chronic hepatitis, which consequently might lead to cirrhosis and hepatocellular carcinoma. The obtained evidence suggests HBV genotype D is associated with reactivation of chronic disease.

COMMENTS

Background

Heterogeneity in the global distribution of hepatitis B virus (HBV) genotypes may account for differences in the clinical outcomes of HBV infected patients and their

responses to antiviral treatment. The clinical and serological statuses of patients infected with HBV of a specific genotype in this geographic region (Iran) need to be further investigated.

Research frontiers

Genotype D was the only type found in different clinical forms of acute and chronic infections. A significant association between the presence of anti-HBe antibody and increased alanine aminotransferase (ALT) levels among HBeAg-negative and HBeAg-positive individuals was demonstrated.

Innovations and breakthroughs

This study suggests the unique characteristics of HBV-D infection are related to geographical location as well as ethnicity. The obtained evidence suggests that HBV genotype D is associated with reactivation of chronic disease.

Applications

Further research should explain the mechanism of pathogenesis of different HBV genotypes and its relation to special geographical region.

Peer review

This is an interesting and well-written clinical epidemiology study on Hepatitis B genotypes in Iran with disease correlation. The paper contributes to the viral hepatitis literature as there is very little from the Middle East in the area of Hepatitis B.

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CLINICAL RESEARCH

Transnasal endoscopic retrograde cholangiopancreatography using an ultrathin endoscope: A prospective comparison with a routine oral procedure

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with less cardiovascular stress and no need of prior EGD or mouth-to-nose transfer of the ENBD tube. However, a deliberate application is needed since its performance is difficult in some cases and is not feasible for some endoscopic treatments such as stenting.

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Key words: Endoscopic retrograde cholangiopancreatography; Nasal endoscopy; Cardiovascular stress; Blood pressure; Sedation; Endoscopic naso-biliary drainage

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Abstract

AIM: To investigate if transnasal endoscopic retrograde cholangiopancreatography (n-ERCP) using an ultrathin forward-viewing scope may overcome the disadvantages of conventional oral ERCP (o-ERCP) related to the large-caliber side-viewing duodenoscope.

METHODS: The study involved 50 patients in whom 25 cases each were assigned to the o-ERCP and n-ERCP groups. We compared the requirements of esophagogastroduodenoscopy (EGD) prior to ERCP, rates and times required for successful cannulation into the pancreaticobiliary ducts, incidence of post-procedure hyperamylasemia, cardiovascular parameters during the procedure, the dose of a sedative drug, and successful rates of endoscopic naso-biliary drainage (ENBD).

RESULTS: Screening gastrointestinal observations were easily performed by the forward-viewing scope and thus no prior EGD was required in the n-ERCP group. There was no significant difference in the rates or times for cannulation, or incidence of hyperamylasemia between the groups. However, the cannulation was relatively difficult in n-ERCP when the scope appeared U-shape under fluoroscopy. Increments of blood pressure and the amount of a sedative drug were significantly lower in the n-ERCP group. ENBD was successfully performed succeeding to the n-ERCP in which mouth-to-nose transfer of the drainage tube was not required.

CONCLUSION: n-ERCP is likely a well-tolerable method

Mori A, Ohashi N, Maruyama T, Tatebe H, Sakai K, Shibuya T, Inoue H, Takegoshi S, Okuno M. Transnasal endoscopic retrograde cholangiopancreatography using an ultrathin endoscope: A prospective comparison with a routine oral procedure. *World J Gastroenterol* 2008; 14(10): 1514-1520 Available from: URL: <http://www.wjgnet.com/1007-9327/14/1514.asp> DOI: <http://dx.doi.org/10.3748/wjg.14.1514>

INTRODUCTION

Ultrathin endoscopy has been developed to perform unsedated transnasal esophagogastroduodenoscopy (EGD) as an alternative procedure to conventional oral endoscopy^[1-6]. Although the evaluation of the ultrathin endoscope has not been established yet^[7-10], it has been suggested that transnasal EGD with an ultrathin scope diminishes the cardiopulmonary complications, lowers the cost because of dispensability of post-procedure monitoring related to consciousness sedation, and improves the patient's acceptability^[11,12]. We have also demonstrated that nasal EGD is better accepted by unsedated patients with less cardiovascular stress compared to conventional oral EGD^[13-15].

Very recently, our preliminary studies have shown further application of an ultrathin upper endoscope to endoscopic retrograde cholangiopancreatography (ERCP) through the nasal^[16] and gastric stomal routes^[17]. Some complications of ERCP including cardiovascular stress may be related to a large-caliber endoscope^[18] and furthermore

a side-viewing scope usually used in ERCP is not feasible for the screening of esophagogastroduodenal tract. Thus, the use of an ultrathin forward-viewing scope for ERCP might be beneficial to lessen those disadvantages. In fact, we have shown that transnasal ERCP with an ultrathin scope decreased cardiovascular stress as compared to the conventional oral method^[16]. Another advantage to use an ultrathin scope is no need of mouth-to-nose transfer of a drainage tube in performing endoscopic naso-biliary drainage (ENBD) because the scope is inserted *via* the transnasal approach^[16]. Moreover, we as well as others have recently reported the direct cholangioscopy by utilizing an ultraslim endoscope for the diagnosis and treatment of choledocholithiasis^[19-21].

Therefore, the application of an ultrathin endoscope to ERCP would be worthy of evaluation. However, limited information is available so far regarding transnasal ERCP with an ultrathin scope. Here, we report a prospective study to estimate the usefulness of an ultrathin scope in performing ERCP comparing to a conventional endoscope. We have used a transnasal method with an ultrathin scope, which may be advantageous in performing ENBD.

MATERIALS AND METHODS

Patients

Fifty consecutive patients, who underwent ERCP for the diagnosis and treatment of the pancreatobiliary disorders in Inuyama Chuo Hospital between March 2006 and March 2007 (Table 1), were enrolled in the present study. The patients were assigned alternately to two groups: 25 patients each underwent either transnasal ERCP with an ultrathin endoscope (n-ERCP group) or transoral ERCP with a conventional scope (o-ERCP group). We excluded the patients with a history of esophagogastrintestinal surgery and cardiovascular diseases and those who were expected to undergo sphincterotomy, papillary balloon dilatation. All patients gave written informed consent before participating into the study. The study was performed in compliance with the Declaration of Helsinki and was approved by the review board for human research of our hospital.

Endoscopy procedure

ERCP was performed by three senior endoscopists with more than 10 years' experience. Two types of endoscopes were used; an ultrathin endoscope (EG530N, Fujinon-Toshiba, Tokyo, Japan with an outer diameter of 5.9 mm, biopsy channel diameter of 2 mm and working length of 1100 mm; forward viewing 120 degree; tip deflection 210 degree up, 90 degree down, and 100 degree right and left) for the transnasal approach and a conventional duodenal endoscope (ED450XT8, Fujinon-Toshiba, Tokyo, Japan with an outer diameter of 13.5 mm) for the oral approach. The nasal cavity was prepared before transnasal ERCP by spraying three doses of 0.111% tramazoline hydrochloride (Alfresa Pharma, Osaka, Japan), followed by the insertion of an 18 Fr catheter covered with 8% lidocaine liquid (Astra Zeneca, Tokyo, Japan) for 3 min. Pharyngeal anesthesia was performed by lidocaine jelly (approximately 5 mL of 2% gel) before the oral endoscopy. Atropine sulfate (0.5 mg) and glucagon (1 USP) were given intramuscularly just before

Table 1 Patient characteristics and baseline cardiovascular parameters in each group (mean \pm SD)

Groups	o-ERCP	n-ERCP	P-value
Number of patients	25	25	
Male/Female	17/8	17/8	0.76
Median age (range)	69 (32-89)	74 (35-85)	0.07
Systolic blood pressure (mmHg)	129 \pm 20	133 \pm 19	0.50
Pulse rate (/min)	71 \pm 12	65 \pm 11	0.08
SpO ₂ (%)	99 \pm 1.6	98 \pm 1.6	0.35
Indication for ERCP (n)			
Cholecyst lithiasis	6	2	
Choledochus lithiasis	11	10	
Cancer	6	10	0.50
Pancreatitis	1	2	
Others	1	1	

O-ERCP: Oral ERCP; n-ERCP: Nasal ERCP.

both procedures. Conscious sedation was carried out by an intravenous injection of midazolam (Astellas, Tokyo, Japan) during both ERCP procedures.

In performing n-ERCP, an ultrathin endoscope was inserted through the nasal cavity and reached the second or third portion of the duodenum after screening observations of the upper gastrointestinal tract. There was no difficulty in observing the papilla of Vater and the cannulation for cholangiopancreatography was performed using a conventional ERCP tube through the biopsy channel of the ultrathin scope as reported previously^[16]. In case of need, an ERCP tube was easily replaced by an ENBD tube (ENBD-5-NAG, Cook, Bloomington, USA) using a guide-wire (Jagwire 0.025/450 Boston Scientific, Natick, USA). Thereafter, the scope was withdrawn carefully through the nasal cavity, leaving the ENBD catheter in the bile duct.

Procedure-related measurements

We have compared between the two procedures the rates of successful cannulation into the common bile duct and/or pancreatic duct, the times required for the first cannulation into either of the ducts, the doses of the sedative drug, the incidence of post-procedural hyperamylasemia and the successful rates of ENBD. The patients with increased serum amylase levels more than twice of normal range after two hours of ERCP were diagnosed hyperamylasemia.

Measurement of cardiovascular responses

Pulse (P), systolic blood pressure (BP) and peripheral blood oxygen saturation (SpO₂) were measured using a monitor unit (DS-7100 Dynascope, Fukuda Denshi, Nagoya, Japan) at the right upper arm. The parameters were determined at rest before the examination, just before the cannulation of the papilla of Vater and at the completion of the procedure. Patients lay quietly in the prone position during the procedure. The rate-pressure product was calculated by a multiplication of BP and P. Changes of the parameters (Δ P, Δ BP, Δ SpO₂ and Δ rate-pressure product) were calculated by the following equation: (values during the endoscopy) - (values before the endoscopy).

Sample size

According to our preliminary study^[16], the required sample

Table 2 Esophagogastroduodenoscopy prior to ERCP and its diagnosis

Groups	o-ERCP	n-ERCP
Number of patients	25	25
EGD prior to ERCP	25	0
Diagnosis		
Normal finding	8	10
Pathological findings	17	15
Reflux esophagitis	1	2
Sliding hernia	4	2
Esophageal diverticulum	1	0
Atrophic gastritis	10	11
Duodenal ulcer scar	1	4
Gastric adenoma	1	1
Gastric SMT	0	1

EGD: Esophagogastroduodenoscopy; SMT; Submucosal tumor.

size was estimated for $\alpha = 5\%$ (two-sided) and $\beta = 10\%$ as well as concerning a risk of cannulation failure in n-ERCP (approximately 30%). With the aim of detecting a standardized difference of 30 mmHg and a standard deviation of 25 mmHg in Δ BP, a sample size of 25 patients per group was needed.

Statistical analysis

Results were expressed by a median value and interquartile as well as 10%-90% quantity range for nonparametric data, and by a mean \pm SD and mean \pm SE for parametric data. Statistical analysis was carried out using Welch-*t* test or paired *t*-test as a parametric test for P, BP, SpO₂ and changes of the parameters. Mann-Whitney-*U* test and chi-squared test were used for as a nonparametric data and categorical data. All analyses were performed with the SPSS for Windows (SPSS Japan, Tokyo, Japan). A *P* value less than 0.05 was considered significant.

RESULTS

Patients' characteristics and procedure-related measurements

No significant difference was found in the gender, age or types of diseases between the two groups (Table 1). Requirement of EGD prior to ERCP is shown in Table 2. It is notable that no prior EGD was needed in the n-ERCP group, whereas EGD was routinely performed before ERCP in all the cases in the o-ERCP group in order to avoid the gastrointestinal injury possibly caused by the side-viewing scope. The diagnoses with the screening EGD involved not a few numbers (approximately 20%) of the esophageal disorders that would be difficult to find by a side-viewing scope (Table 2).

Procedure-related measurements are presented in Table 3. We have successfully performed n-ERCP in 23 of 25 cases (92%) except two cases with gallbladder cancer and choledocholithiasis due to the insertion failure of the endoscope into the second portion of the duodenum. In the n-ERCP group we have completed the cannulation into the common bile duct in 18 of 25 cases (72%) and into the pancreatic duct in 21 of 23 attempted cases (91%).

Table 3 Successful rates of cannulation and endoscopic naso-biliary drainage and rates of post-ERCP hyperamylasemia in each group

Groups	o-ERCP	n-ERCP	<i>P</i> -value
Successful cannulation (%)			
Either of the ducts	25/25 (100)	23/25 (92)	0.510
Common bile duct	24/25 (96)	18/25 (72)	0.053
Pancreatic duct	24/25 (96)	21/23 (91)	0.940
Median time for cannulation, min (%)	8 (2-24)	7 (4-20)	0.590
Post-procedural hyperamylasemia (%)	7/25 (28)	5/23 (22)	0.870
Successful ENBD (%)	20/21 (95)	13/15 (87)	0.340

O-ERCP: Oral ERCP; n-ERCP: Nasal ERCP; ENBD: Endoscopic naso-biliary drainage.

There was no difference in the procedure time required for the cannulation between the groups (Table 3).

Although no significant difference was found in the rate of successful cannulation between the two groups, there was a tendency to a lower success rate of cannulation into the common bile duct in the n-ERCP group (Table 3). Therefore, we have further analyzed the relationship between the successful insertion rate and the patterns of endoscopic shapes during the approaches to the papilla of Vater. We have classified the manners of approaches into three categories: C, U and J patterns according to the endoscopic shape observed under fluoroscopy (Figure 1). In the C and U patterns the scope was inserted along the duodenal loop in the second and third portions. In the C pattern a front view of the papilla of Vater was observed easily, whereas in the U pattern its observation was more difficult, where the papilla could only be looked up from the third portion of the duodenum (Figure 1). The J pattern was the reverse shape of the U loop. The papilla of Vater was also looked up from the downward in the J pattern, however, its view was obtained more easily than in the U pattern. Favorable results were obtained in the C and J patterns regarding both the cannulation rates and its required times (Table 4). However, because in the U pattern the angle to observe the papilla of Vater was sharpest, we had difficulty in the cannulation, resulting in the lowest cannulation rate into the common bile duct as well as the longest time for cholangiography among the patterns (Table 4).

Post-procedural hyperamylasemia was found in 22% and 28% in the n-ERCP and o-ERCP groups, respectively, which was not significantly different (Table 3). Pancreatitis was not encountered in either procedure, suggesting no disadvantage of n-ERCP regarding procedure-associated complications. Interestingly, the dose of a sedative drug (midazolam) required during the procedure was significantly smaller in the n-ERCP group than in the o-ERCP group ($P = 0.003$, Figure 2). Insertion of an ENBD tube was attempted in 15 cases in the n-ERCP group and was successfully performed in 13 cases (87%), except two cases with the incomplete deep cannulation into the common

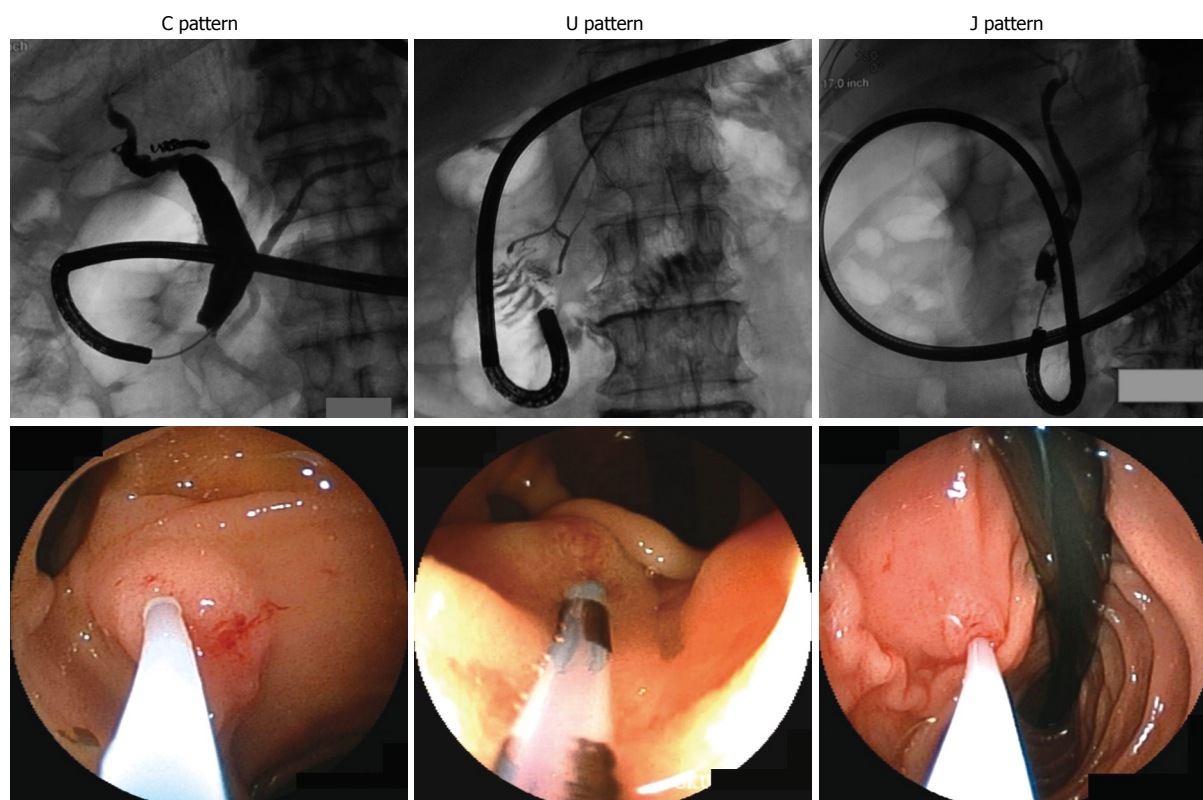


Figure 1 Patterns of the endoscopic shape under fluoroscopy in performing transnasal ERCP. Upper panels, fluoroscopic views of the C, U and J shapes of an ultrathin scope; Lower panels, endoscopic views of the papilla of Vater observed in the respective patterns.

Table 4 Rates and times required for successful cannulation in each pattern of the endoscopic shape

Patterns	C	U	J	Total
Successful cannulation (%)				
Either of the ducts	6	14	3	23
Common bile duct	6/6 (100)	9/14 (64)	3/3 (100)	18/23 (78)
Pancreatic duct	5/5 (100)	14/14 (100)	2/2 (100)	21/21 (100)
Median time for cannulation, min (%)				
Either of the ducts	6.5 (5-8)	7.3 (4-20)	7 (4-14)	7 (4-20)
Common bile duct	10.5 (8-37)	20 (7-35.5)	16 (6-19)	17 (6-37)
Pancreatic duct	6 (5-8)	13 (6-26)	6 (5-7)	9 (5-26)
Termination	31 (14-38)	34 (11-61)	24 (12-34)	33 (11-61)

bile duct (Table 3). No significant difference was found in the successful rate of ENBD between the groups.

Measurement of cardiovascular responses

There was no significant difference in the baseline cardiovascular parameters measured before ERCP between the n-ERCP and o-ERCP groups (Table 1). Both Δ BP and Δ rate-pressure product measurements just before the cannulation of the papilla of Vater and at the end of ERCP procedure were significantly smaller ($P < 0.001$, respectively) in the n-ERCP group than in the o-ERCP group (Figure 3A and C). There was no significant difference in Δ P (Figure 3B)

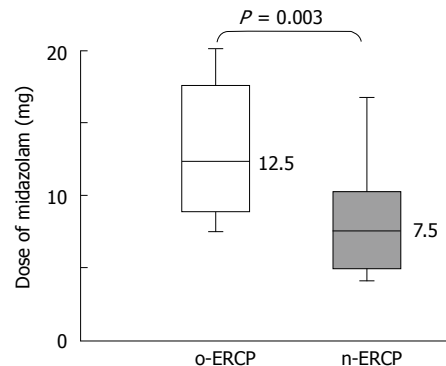


Figure 2 Dose of midazolam, a sedative drug used in ERCP. Values represent the mean, interquartile and 10%-90% quantities. O-ERCP: Oral ERCP with a conventional scope; N-ERCP: Transnasal ERCP with an ultrathin scope.

and Δ SpO₂ (Figure 3D) between the two groups both just before the cannulation and at the end of ERCP.

DISCUSSION

ERCP is a widespread technique essential for the diagnosis and treatment of the pancreatobiliary disorders. Although ERCP is an established method, there are still several disadvantages in its performance including the procedure-related morbidity^[11]. Here, we have focused upon the following unfavorable aspects of the conventional ERCP: (1) a side-viewing duodenoscope is not suitable for the screening observation of the esophagogastrroduodenal tract prior to performing ERCP, (2) a large caliber scope may exacerbate the cardiovascular stress, and (3) transfer

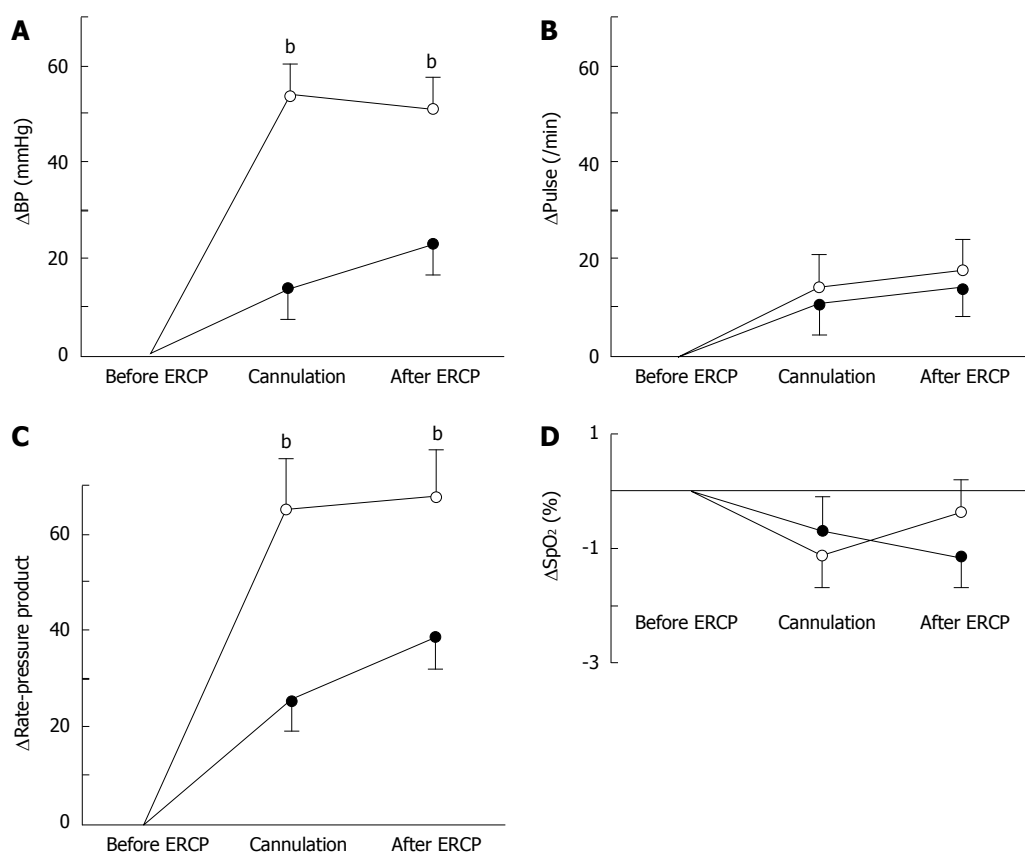


Figure 3 Cardiovascular parameters during the ERCP procedure. **A-D** show the changes in systolic blood pressure (**A**), pulse rate (**B**), rate-pressure product (**C**) and peripheral blood oxygen saturation (**D**). The changes of the parameters were calculated as described in the text. Open and closed circles show the oral and transnasal ERCP, respectively. Each parameter was measured before performing ERCP, just before the cannulation and at the completion of the ERCP procedure. Values represent the mean \pm SE. ^b $P < 0.001$.

of a drainage tube from the oral to nasal cavity is necessary in performing ENBD. We have investigated in the present study if those disadvantages could be overcome by the transnasal approach employing an ultrathin forward-viewing endoscope.

Most of the patients with acute pancreatobiliary disorders have the symptoms indistinguishable from those of the esophagogastrintestinal diseases and in fact the gastrointestinal complications are often encountered in such patients. Therefore, the observation with EGD prior to ERCP is preferable, as we have routinely performed in the present study. However, since a forward-viewing scope employed for n-ERCP is usually used to examine the upper gastrointestinal tract, additional EGD was not necessarily performed prior to n-ERCP. Simultaneous screening of the gastrointestinal tract may not only shorten the time required for the diagnosis but also assist performing safer ERCP by reducing a risk of the esophagogastrroduodenal injuries that might occur during the conventional ERCP procedure with a side-viewing scope. Moreover, a forward-viewing scope has an advantage in examining the esophageal diseases that would be hardly diagnosed by a side-viewing scope. In fact, not a few numbers of esophageal diseases were found in the present study.

A large-caliber endoscope has been suggested to exacerbate cardiovascular stress^[18]. Here, we have also shown that n-ERCP with an ultrathin scope reduced the cardiovascular stress as we have reported previously^[16]. A significantly lower increment of the rate-pressure product in n-ERCP may strongly support the reduction of the risk of cardiovascular complications as compared to conventional o-ERCP^[22]. Furthermore, we have

found that n-ERCP required a significantly lower dose of the sedative drug than o-ERCP. These observations suggest the reduced physical as well as presumably less mental stress in performing n-ERCP. Previous studies have suggested that the sedative used during EGD is an important factor in the genesis of cardiopulmonary complications and thus milder sedation could diminish its incidence^[11,23]. Therefore, a lower requirement of a sedative drug in n-ERCP might also be advantageous to lessen the cardiovascular complications. In fact, n-ERCP with an ultrathin endoscope has been anticipated being advantageous to reduce myocardial ischemia because of its less mechanical irritation^[24-27].

A guideline for the diagnosis and treatment of acute cholangitis and cholecystitis has recently been proposed in which an early biliary drainage is strongly recommended^[28]. ENBD is a valuable and safe technique for the urgent biliary drainage. We have successfully performed ENBD in succession to n-ERCP without any complications. Because there is no need of EGD prior to ERCP, n-ERCP may be valuable in performing urgent ENBD. Another advantage of transnasal approach is no need to transfer the ENBD tube from oral to nasal cavity.

Those observations in our study suggest that n-ERCP is a well-tolerable technique that may overcome some disadvantages of conventional o-ERCP. However, it should be noted that there are significant limitations in performing n-ERCP due to a narrow channel of the scope. For instance, the technique is not feasible for sphincterotomy, papillary balloon dilatation, and stenting using a large-size stent with a diameter of 5 Fr or more. Therefore, we do understand the necessity and widespread

utility of o-ERCP, and thus we do not intend to emphasize excessively the usefulness of n-ERCP. Moreover, in some cases n-ERCP is difficult to perform and thus would be better replaced with o-ERCP. Particularly, in cases with the U pattern of the endoscopic shape the successful cannulation rate was relatively low and requires a longer time, which might lead to an increased risk of the procedure-related complications such as acute pancreatitis^[29] and ischemic heart injury^[26]. Therefore, we propose the following strategy to perform n-ERCP in the clinical practice. In cases who are not expected to undergo sphincterotomy or papillary balloon dilatation, transnasal EGD is routinely performed prior to ERCP using an ultrathin scope that usually reaches the papilla of Vater. Then, when the approach pattern is C or J under fluoroscopy the cannulation for cholangiopancreatography seems to be easily and safely performed and thus n-ERCP may be attempted. However, in cases with the U pattern the scope may better be replaced with a conventional side-viewing duodenoscope when an endoscopist had difficulty in the cannulation. In addition, we have experienced successful n-ERCP in several cases in whom the papilla of Vater was hard to observe using a side-view scope and thus we failed to perform o-ERCP. N-ERCP may also be a good indication in such cases.

Future advancement of ERCP techniques using an ultrathin scope might allow the minimally invasive interventions that otherwise can hardly be carried out by conventional ERCP. For example, endoscopic diagnosis and therapy for the pancreatobiliary disorders may extend to the elderly as well as to the patients with difficulties in oral insertion, including those with esophagogastrintestinal stenosis and gastric stoma as we have reported previously^[17]. Very recently, we have successfully performed lithotomy under direct cholangioscopy utilizing the same ultrathin scope used in the present study^[19,20]. Thus, it is possible to remove a biliary stone in succession to n-ERCP in cases who had undergone sphincterotomy. Future development of an ultrathin duodenoscope designed especially for ERCP would bring the wide spread of the transnasal ERCP.

COMMENTS

Background

Conventional oral endoscopic retrograde cholangiopancreatography (ERCP) has several disadvantages related to a large caliber side-viewing duodenoscope, including the cardiovascular stress caused by a large-caliber scope, difficulty in observing the esophagogastrroduodenal tract prior to performing ERCP, and necessity of mouth-to-nose transfer in performing endoscopic naso-biliary drainage (ENBD). Transnasal upper endoscopy using an ultraslim scope has been shown to be better tolerated with less cardiovascular stress.

Research frontiers

Very recently, our preliminary studies have shown the better cardiovascular tolerance of transnasal ERCP using an ultraslim forward-viewing scope as well as an application of the scope to direct cholangioscopy. The highlight of the present study is to demonstrate the feasibility of the ultrathin scope in performing ERCP, comparing with conventional oral ERCP.

Innovations and breakthroughs

Little information has been available regarding the application of an ultrathin upper endoscope to ERCP. The present study is the first report showing the utility and safety of transnasal ERCP with an ultraslim scope. We have demonstrated that

nasal ERCP can be performed safely with similar successful rates of cannulation into the pancreatobiliary ducts as compared with those of the conventional method, and that nasal ERCP is less stressful to the cardiovascular system and is advantageous in performing ENBD due to no necessity of mouth-to-nose transfer of a drainage tube.

Applications

ERCP using an ultrathin scope may allow endoscopic diagnosis and therapy for the pancreatobiliary disorders in patients with difficulties in insertion, including those with esophagogastrroduodenal stenosis and gastric stoma. Future development of the ultrathin duodenoscope might bring the widespread of transnasal ERCP.

Peer review

The present study is the first demonstration of the feasibility and safety of transnasal ERCP utilizing an ultraslim upper endoscope. The findings are novel and the study design is well organized.

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Regulatory T cells in patients with inflammatory bowel diseases treated with adacolumn granulocytapheresis

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Abstract

AIM: To investigate if the clinical efficacy of granulocytes and monocytes by adsorption (GMA) is associated with an increased frequency of peripheral regulatory T cells (Tregs), as these cells have proven to be successful in suppressing inflammatory bowel disease (IBD) in animal models.

METHODS: We report four cases of corticosteroid-dependent ulcerative colitis (UC) and two Crohn's disease (CD) cases with severe cutaneous lesions who received GMA therapy. The frequency of CD4⁺ CD25^{high} (Tregs) in peripheral blood was analyzed by flow cytometry and the expression of FoxP3 and TGF beta in purified CD4⁺ T cells was determined by real time PCR prior to and one month after the last apheresis session, and at the time of endoscopic and clinical assessing.

RESULTS: Increased expression of Fox P3 mRNA was found in all five patients who responded to cytapheeresis with remission of clinical symptoms, mucosal inflammation and cutaneous lesions, and an increased frequency of circulating Tregs was found in four patients. These changes were not observed in the patient with UC who did not respond to GMA. Variations in TGF-β (mRNA) did not parallel that of FoxP3 mRNA.

CONCLUSION: The clinical efficacy of GMA on IBD and related extra intestinal manifestations was associated with an expansion of circulating CD4⁺ CD25⁺ Tregs and higher expression of FoxP3 in CD4⁺ T cells. Accordingly, an elevated CD4⁺ CD25⁺ FoxP3 may be a valuable index of remission in patients with IBD and other chronic relapsing-remitting inflammatory conditions during treatment with GMA.

INTRODUCTION

Granulocyte-monocyte adsorption apheresis (GMA) with the Adacolumn has shown efficacy in the treatment of inflammatory colitis in a series of studies, mainly performed by Japanese and European investigators^[1,2]. Adacolumn[®] is a therapeutic extracorporeal device, which selectively adsorbs granulocytes and monocytes from peripheral blood. The selective adsorption is thought to be mediated by interactions of complement and Fc receptors on the granulocytes and monocytes, whereas complement factor and immunoglobulins adhere onto the surface of G-1 beads by ligand-receptor interactions^[3]. Granulocytes and monocytes are believed to play a significant role in these and other autoimmune inflammatory diseases because of their capability to release a variety of mediators including active oxygen species and inflammatory cytokines, such as tumor necrosis factor-α (TNF-α) and interleukin β (IL1-β)^[4,5]. This contention supports the use of GMA-apheresis and other therapeutic attempts to prevent damages caused by granulocytes and monocytes^[6,7]. However, the clinical efficacy of cytapheeresis cannot be fully explained by a simple transient removal of these cells.

CD4⁺ CD25^{high}, FoxP3⁺ regulatory T cells (Tregs) play a major role in controlling the immune response by producing suppressive effects on activated immune cells. It is established that FoxP3, a forkhead/winged-helix transcription factor, is specifically expressed in Treg cells and is a master control protein for the generation of these cells^[8]. Depletion of Tregs leads to autoimmunity in mice^[9] and dysfunction of Tregs has been linked to human autoimmune diseases^[10-13]. Immunosuppressive cytokines like IL-10 and

TGF- β are required for the control of autoimmunity in many *in vivo* models, but suppression *in vitro* appears to be solely mediated by a cell-cell contact-dependent, cytokine independent mechanism^[14]. Further, an inverse correlation has been reported between disease activity and frequency of peripheral Tregs in patients with inflammatory bowel disease (IBD)^[15]. However, active IBD is not associated with a functional defect but with a contraction of the peripheral blood Treg pool and a moderate expansion in intestinal lesions^[16]. Further, cultured gut-derived Tregs from patients with Crohn's disease (CD) and healthy individuals suppress T cell proliferation and cytokine secretion^[17]. Animal studies suggest that adoptive transfer of Tregs can reverse experimental colitis^[18] and the long-term effects of *ex vivo* activated and transferred Treg cells are likely due to their ability to generate new cytokine-producing CD4+ Treg *in vivo*^[19].

The involvement of TGF- β in Treg function remains controversial but it has been reported that TGF- β produced by CD4+ CD25+ T cells is involved in the suppressor activity of these cells, particularly in their ability to regulate intestinal inflammation^[20]. There is evidence that TGF- β deficiency leads to development of autoimmune disease: TGF- β deficient mice and transgenic mice expressing the dominant negative form of TGF- β receptor are useful animal models for diabetes and IBD^[21,22]. Suppressive activity of Tregs may be inhibited in TGF- β deficiency by acting on FoxP3. TGF- β is able to induce FoxP3 expression and subsequently a Treg phenotype in CD4+CD25- murine and human T cells, as well as a positive auto regulatory loop of TGF- β signaling by down-regulation of smad-7, an inhibitory protein for TGF- β signaling^[23]. In addition, over expression of smad 7 that results in insensitivity to TGF- β has been associated with human IBD^[24].

With the assumption of a role of Tregs in the prevention and potential cure of autoimmune inflammatory diseases, we aimed to ascertain whether the beneficial effects of GMA-apheresis correlate with an increase in CD4+ CD25^{high} cell numbers and a higher expression of FoxP3 and TGF- β in peripheral blood CD4+ T cells in a small group of patients with ulcerative colitis or Crohn's disease.

MATERIALS AND METHODS

Subjects

Four patients with ulcerative colitis (UC) and two patients with CD that were treated by selective GMA were included in this study. Main UC patient's data are presented in Table 1. Steroid dependency was defined as the need for > 10 mg prednisolone daily for at least 4 wk to control clinical symptoms. Both of the two patients with CD had associated severe cutaneous conditions; patient 5, a 26 aged female was diagnosed with CD 7 years ago. This patient had ileocolonic lesions without fistulas and had previously been treated with steroids, 5-ASA, and azathioprine cycles without improvement. On August 2005, she had an ulcerative lesion in the left leg; diagnosis of pyoderma gangrenosum (PG) was made on clinical grounds and confirmed by a dermatologist. A skin biopsy was not required as there was no clinical doubt in the diagnosis.

Patient 6 was a 45-year old female who was diagnosed to have CD with ileocolonic inflammation and has had several flares of uveitis and nodosum erythematosis. She was unsuccessfully treated with steroids and anti-TNF antibodies.

Each patient received five cycles GMA-apheresis at weekly intervals (see below) and a clinical protocol including ileocolonoscopy was applied one month after the end of the therapy. Clinical outcome was assessed using the follow-up criteria: absence/near absence of clinical symptoms as remission; improvement of symptoms, (without increase in steroid dose) as response, and no change or worsening as failure. Disease activity was categorized as mild, moderate or severe, and relapse was defined as an increase in clinical symptoms, as judged by the treating physician. Blood samples were taken at entry and when efficacy was assessed to determine the frequency of peripheral regulatory CD4+ CD25^{high} T cells and to quantify mRNAs for FoxP3 and TGF- β expressed in purified CD4+ T cells. Results from each individual patient were compared regarding to its clinical response to the therapy (Table 2).

GMA procedures

Patient's blood was passed through a column that is filled with specially designed cellulose acetate beads of 2 mm in diameter that absorb about 65% of granulocytes, 55% of monocytes and a very small fraction of lymphocytes^[25]. The apheresis procedures were performed by a specially trained nurse at the outpatient gastrointestinal department. Adacolumn device (JIMRO, Takasaki, Japan) was used in all cases. The GMA sessions were performed at a flow rate of 20-30 mL/min for 60-90 min and the frequency and total number of apheresis sessions were typically 5 weekly sessions per patient. Non-adverse events or toxicity were observed.

Flow cytometry

Whole blood samples were stained with FITC (fluorescein) labeled anti-CD4 and APC conjugated anti-CD25 Abs; respective mouse isotype controls were employed; optimal dilution of antibodies were used and cells were washed and analyzed by flow cytometry in a FACScalibur cytometer and the CellQuest software (Becton Dickinson). A live gate set around viable lymphocytes was used based on their forward scatter/side scatter (FCS/SCC) characteristics. For analysis of CD25^{high} T cells, large activated cells as determined by FCS/SCC properties were excluded. Three cell populations were distinguished in CD4+ T cells, according to the surface expression of CD25: CD4+ CD25- "naïve"; CD4+ CD25^{low-int} "activated", and CD4+ CD25^{high} "regulatory" cells.

Cell isolation

Peripheral blood mononuclear cells (PBMC) were isolated from 20 mL freshly drawn blood by Ficoll density gradient centrifugation. CD4 positive cells were isolated from PBMC by positive selection using anti-CD4 human micro beads magnetic adhesion cell separator (MACS) antibody (Miltenyi Biotech, Bergisch, Gladbach, Germany).

Table 1 Ulcerative colitis patient's data

	1	2	3	4
Age (yr)	37	42	38	36
Sex	Male	Female	Female	Male
Disease' location	Left side	Extensive colitis	Left side	Left side
Disease activity	Moderate-severe	Severe	Severe	Severe
Time from diagnosis (yr)	2	10	8	22
Truelove index	13	17	14	15
Previous therapy	Corticosteroids azathioprine	Corticosteroids azathioprine	Corticosteroids azathioprine	Corticosteroids azathioprine
Indication for GMA-apheresis	Steroid-dependent disease	Steroid-dependent disease	Steroid-dependent disease	Steroid-dependent disease
Cycles of GMA-apheresis	10	5	7	5
Response to GMA-apheresis	Remission	Response	Failure	Remission
Truelove index (after therapy)	10	10	16	9
Endoscopy	No inflammation	No inflammation	Bleeding ulcers	No inflammation
Recurrences	At 6 mo	At 6 mo		Non

GMA: Granulocyte/monocyte apheresis.

Quantitative PCR

CD4+ cell pellets were lysed in RLT buffer (Qiamp RNA blood mini kit (Qiagen, Germany) and total RNA was extracted. One microgram of total RNA was reverse transcribed using MMLV and random hexamers (Promega, USA) at 42°C one hour, and at 65°C 10 min. The cDNA obtained was stored at -20°C until PCR analysis.

FoxP3 and glucuronidase (GUS) as a housekeeping gene were analyzed by quantitative real time PCR performed with the Light Cycler Fast Start DNA Sybr green kit (Roche). FoxP3 and GUS primers and PCR conditions are described in^[26] and^[27] respectively. Reverse 3'primer are designed to span contiguous exons in order not to anneal to contaminating genomic DNA. To control for specificity of the amplification products, a melting curve analysis was performed and no unspecific products were observed. A standard curve of cDNA from CD4 cells from a donor with a high expression of FoxP3 was used. Arbitrary units were assigned to each dilution of cDNA. Relative FoxP3 expression values were calculated dividing the arbitrary units of Fox P3 by the GUS results in order to normalized RNA input. The result is expressed in % ratio.

TGF-β1 and human-glucuronyl-6-phosphatase dehydrogenase (h-G6PDH) as a housekeeping gene were analyzed by quantitative real time PCR performed with Light Cycler Fast Start DNA hybridization probes kit (Roche). Primers and probes specific to TGF-β1 isoform were: Forward: exon 6: 5'-ggCTggAAgTggATCCACgA-3' Reverse: 5'-gCaggAgCgCACGATCATgTT-3'; Probes: 5'-CTgCCCCTACATTTggAgCCTggAC-F-3' 5'-LCRed640-gCagTACAgCCA gCagTACAgCAAgTCCTggCCCT-P-3'. Reverse 3'primer is designed to span contiguous exons in order not to anneal to contaminating genomic DNA. PCR conditions for both gene amplification were: 45 cycles of 3 segments: 95°C 10 s; 58°C 15 s and 72°C 15 s. A melting curve analysis was performed and no unspecific products were observed. The copy number of TGF-β1 gene was calculated from a standard curve of a plasmid with the TGF-β1 PCR fragment cloned in the laboratory and h-G6PDH copies were determined with

Table 2 Clinical and analytical data of Crohn disease's patients

	Patient 5		Patient 6	
	Pre apheresis	Post apheresis	Pre apheresis	Post apheresis
CDA I	241	161	236	156
Cutaneous lesion	Pyoderma gangrenosum	Healing ¹	Erythema nodosum	Healing ¹
Hematocrit	37.5	37.1	32.3	34
Haemoglobin	11.9	11.9	10.6	10.9
GSV	62	39	74	58
C-reactive protein	67	13	17	4
Platelets × 10 ³ /mL	241	161	236	151

¹Number of GMA-apheresis sessions to achieve the healing: 13 for PG, 5 for EN.

LC-h-G6PDH housekeeping gene set (Roche). Absolute quantification was expressed as ratio % for each cDNA.

RESULTS

Increase in peripheral Treg in patients with IBD who showed a favorable response to GMA

Regulatory T cells are usually identified by flow cytometry as CD4+ T cells expressing high level of the IL2-R α chain (CD25) at their surface (Figure 1). By using this criterion we found higher frequencies in samples obtained at the end of the GMA-apheresis sessions in three of five patients with IBD that responded favorably to the therapy, but a lower frequency in the one who failed to respond to this treatment. To exclude that changes in the frequency of Tregs reflect nonspecific effects of the therapy on other CD4+ T cell subsets, we also calculated the ratio CD4+ CD25^{high} to CD4+ CD25^{low-int} and compared the data in each individual case; higher ratio was found in one case, lower ratio in another, and no variation in the remaining four cases. Thus, a selective expansion of true Treg cells in the peripheral blood of treated patients could not be demonstrated. Human CD4+ CD25^{high} Treg population is

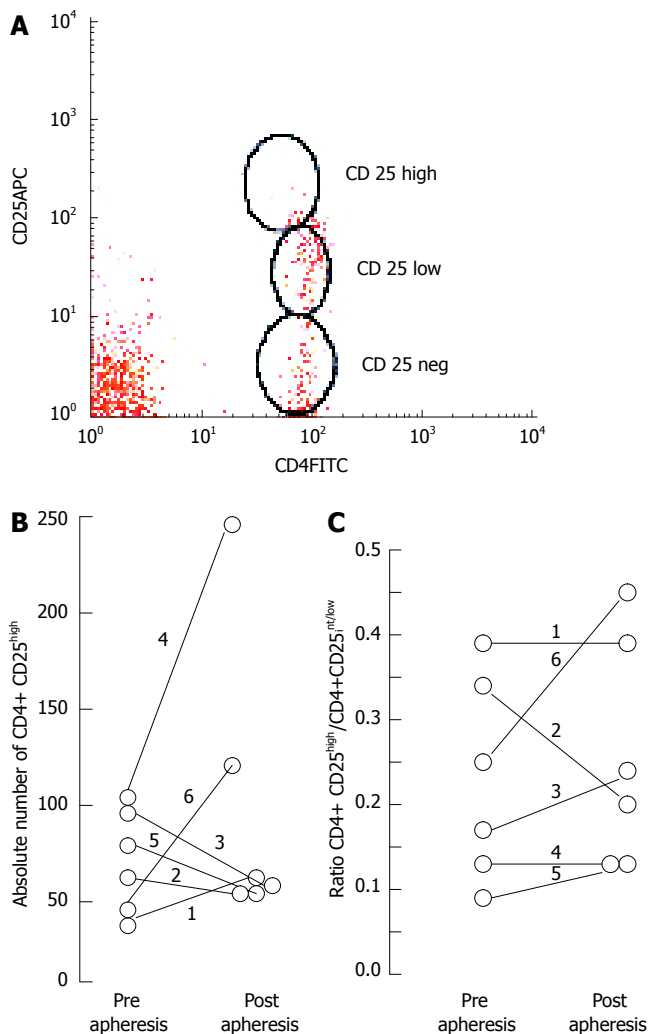


Figure 1 Variation of peripheral blood CD4+ CD25+ cells in patients treated by GMA-apheresis. **A:** Representative cytogram; **B:** Variation of T regs on each individual patient; **C:** Variation in the ratio CD4+ CD25^{high}/CD4+ CD25^{low-int}.

relatively indiscreet; the border line between CD25^{high} and CD25^{low} is often obscure, making it difficult determining the cellular subsets. Even carefully selected CD4+ CD25+ T cell clones do not represent a homogenous population of functional suppressor cells^[28]. This fact may explain the differences in normal ranges described in the literature but it seems unlikely that its concerns the variations observed in this study.

Clearer results were found by quantification of mRNA for FoxP3 expressed in purified CD4+ T cells by real time PCR. In contrast to most phenotypic markers of Tregs, the expression of FoxP3 seems to be crucial for their functional characteristics^[29]. All five patients which showed successful response to Adacolumn therapy displayed increased levels of FoxP3 mRNA in blood CD4+ T cells after the GMA treatment, whereas diminished levels of FoxP3 transcripts were found in the one that did not respond to this therapy (Figure 2).

TGF- β expression in CD4+ T cells does not change after GMA

Next, we analyzed the expression of TGF- β in CD4+ T cells from GMA- treated patients and observed increased

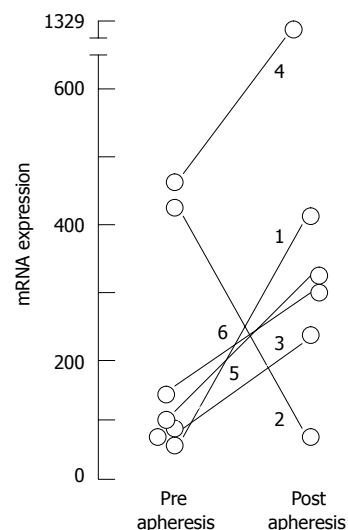


Figure 2 Variation of mRNA expression for FoxP3 in CD4+ T cells in matched samples from 6 patients treated by GMA-apheresis: Quantitative PCR was performed as described in methods; Results are expressed as FoxP3 expression relative to GUS expression per cent.

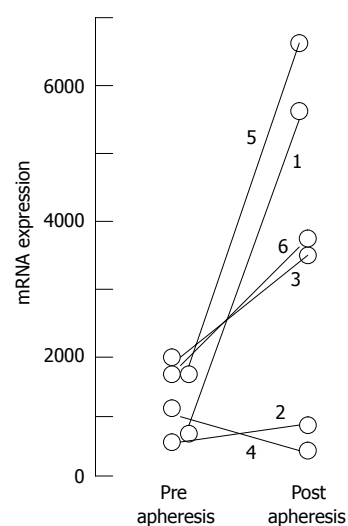


Figure 3 Variation of mRNA expression for TGF-beta in CD4+ T cells in matched samples from 6 patients treated by GMA-apheresis: Quantitative PCR was performed as described in methods; Results are expressed as TGF-beta 1 expression relative to GAPDH expression per cent.

expression of TGF- β in post apheresis samples in four cases but this variation was not parallel to that observed for FoxP3 expression. The remaining two cases did not show variations in TGF- β and have had a different clinical response to GMA-apheresis (Figure 3).

Adacolumn therapy was successfully applied to healing cutaneous lesions related to inflammatory bowel disease

Both two patients with Crohn's disease had severe cutaneous lesions that improved with the Adacolumn's therapy; Figure 4 shows the complete healing of pyoderma gangrenosum, unsuccessfully treated with steroids, after 13 sessions with GMA-apheresis. A successful response to GMA-apheresis was also observed in the case of erythematous nodosum associated with CD that was unsuccessfully treated with steroids and infliximab anti-TNF- α therapy. The effect of apheresis was transient as the cutaneous lesions recurred four months after the interruption of therapy and remitted with a new cycle of GMA-apheresis sessions.

DISCUSSION

A current view is that a dysregulated immunity in



Figure 4 Improvement of pyoderma gangrenosum in a patient with Crohn's disease treated by GMA-apheresis: pictures A-D, were taken at 1, 5, 8, and 13 apheresis sessions.

intestinal mucosa plays a pivotal role in the pathogenesis of IBD and that intestinal inflammation is perpetuated by inflammatory cytokines like IL-1 β , TNF- α , IL6 and others^[30]. Based on this perception, anti cytokine antibodies, notably anti TNF (infliximab) have been added to the conventional anti inflammatory drugs. Steroids, 5-aminosalicylic or sulphasalazine in combination with azathioprine and nutritional support for some patients are used as first-line medication for IBD. However, treatment failure has often been an indication for colostomy in steroid-refractory patients^[31] and biologicals are dampened by serious concerns about their long-term efficacy and safety profiles^[32,33]. The underlying rationale for GMA-apheresis is the fact that granulocytes and monocytes/macrophages (GMs) are a major source of inflammatory cytokines^[34] and that selective removal of these cells, otherwise destined for migration to the intestine, reduce the intestinal inflammation. GMs removal is made by their adhesion to carrier beads through Fc γ R and complement receptors, but it is reported that Adacolumn increases peripheral blood lymphocytes, sparing prevailing cells and inducing an increase in *de novo* lymphocytes^[35], largely attributable to an increase in CD4+ T cells. These changes on blood lymphocytes were also observed in our patients (data not shown). It is noteworthy that a low lymphocyte count has been associated with relapse of CD^[36].

A selective increase of peripheral CD4+ Treg cells in patients treated with GMA has been suggested^[37] and our results strongly support this opinion. We have observed higher frequency of Tregs and increased expression for FoxP3 mRNA in the blood of IBD patients treated with Adacolumn except in the one that did not respond to the

therapy. Quantitative data showed expression differences in matched samples of purified CD4 T cells, obtained before and after a series of GMA. We think that these results contribute to enlighten the picture hence there is no published data at this time.

The position of GMA-apheresis in the medical therapy of ulcerative colitis has been recently reviewed^[38]. According to the expressed opinion patients at any stage of their disease might benefit from this therapy and can avoid steroids and other drug based medications. In patients with steroid dependent UC up to 85% show efficacy with an excellent safety profile. Five from our six patients with active and severe IBD responded to GMA-apheresis therapy and the one that did not respond showed a further favorable response to the infliximab. Pyoderma gangrenosum (PG) is a chronic ulcerating skin condition that appears to be immune mediated, and approximately 30% of cases occur in association with IBD^[39]. The mainstay treatment remains immunosuppression with corticosteroids and cyclosporine, and there is a number of reports of PG responding to anti TNF- α therapy^[39]. We report the beneficial effect of GMA-apheresis therapy in a case of PG and a case of erythema nodosum, both associated with CD that did not respond to other therapies.

Thus, many patients with IBD likely benefit from GMA-apheresis therapy, potentially by an increase in frequency and function of CD4 Tregs that takes place in the action mechanism(s) of this procedure. The small number of patients in our study precludes to take out any statistical conclusion about this point but, independently of defining the actual mechanism of GMA-apheresis in IBD therapy, our results suggest that quantification of

FoxP3 mRNA expressed in CD4 T cells of treated patients may be used as a valuable index of remission in IBD and perhaps in other chronic relapsing-remitting inflammatory conditions susceptible of this kind of therapy. If that were proved on a large series of GMA-treated patients, it could be helpful to predict the response to GMA and to support the rational use of this high-cost therapy. We also point out the fact that the patient that did not respond to GMA did respond to infliximab, and conversely, those which responded to GMA did not respond to other therapies. Thus, a tailor's patient therapy could be considered in a future and good analytical criteria of response will be welcome.

COMMENTS

Background

Granulocyte/monocyte apheresis with Adacolumn seems to offer a safe and efficacious adjuvant treatment option in inflammatory bowel disease (IBD), rheumatoid arthritis and maybe in other inflammatory conditions, but the clinical efficacy cannot be explained by selective removal of these cells. Regulatory T cells play a major role in control of autoimmune diseases, and the FoxP3 transcription factor is specifically expressed in these cells and is crucial for their functional characteristics. Dysfunction of T regs it is suggested in patients with IBD and demonstrated in animal models of these pathologies

Innovations and breakthroughs

We report that the clinical efficacy of adsorptive cytophoresis in the treatment of inflammatory colitis is associated to an expansion of circulating T reg and increased expression of FoxP3. If that were proved in a large series of treated patients it could be helpful to make a valuable index of remission and to predict the response to this therapy.

Applications

All the findings of our study will provide a useful way for the treatment of inflammatory colitis.

Peer review

This is an interesting study. Authors showed that GMA induces increase of Treg and exerts anti-inflammatory response in IBD patients.

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RAPID COMMUNICATION

Increased basolateral sorting of carcinoembryonic antigen in a polarized colon carcinoma cell line after cholesterol depletion-Implications for treatment of inflammatory bowel disease

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Abstract

AIM: To investigate a possible increase of basolateral expression of carcinoembryonic antigen (CEA) by interfering with the apical transport machinery, we studied the effect of cholesterol depletion on CEA sorting and secretion.

METHODS: Cholesterol depletion was performed in polarized Caco-2 cells using lovastatin and methyl- β -cyclodextrin.

RESULTS: We show that CEA is predominantly expressed and secreted at the apical surface. Reduction of the cholesterol level of the cell by 40%-50% with lovastatin and methyl- β -cyclodextrin led to a significant change of the apical-to-basolateral transport ratio towards the basolateral membrane.

CONCLUSION: As basolateral expression of CEA has been suggested to have anti-inflammatory properties, Cholesterol depletion of enterocytes might be a potential approach to influence the course of inflammatory bowel disease.

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Key words: Inflammatory bowel disease; Cholesterol; Polarized secretion; Carcinoembryonic antigen; Caco-2

INTRODUCTION

Carcinoembryonic antigen (CEA, also named CEACAM5, gp180 or CD66e) is a 180 kDa glycoprotein that belongs to a subfamily of the immunoglobulin superfamily. In the intestine, CEA is mainly localized at the apical membrane of the goblet cells and colonic enterocytes^[1]. Although CEA has been used extensively as a tumor marker for colon carcinoma, its physiological function remains unclear. The induction of CEA by cytokines^[2,3], its *in vitro* cell-cell adhesion properties^[4] and its inhibitory effect on natural killer cells^[5] have suggested a significant role on immune function. It has been shown that apically expressed CEA can bind to several microorganisms like *E.coli*, *Salmonella*, *Neisseria* or *Haemophilus*, and possibly function as a sensor to trigger or prevent bacterial infection^[6-10]. On the other hand, basolateral expression of CEA has been shown to act as a CD8 ligand that is involved in the activation of suppressor T cells^[11]. Interestingly, patients with inflammatory bowel disease (IBD) show a significantly reduced expression of CEA regardless of the activity of the disease^[12,13]. Therefore, it has been suggested, that reduced expression of CEA may be an inherited defect in IBD^[13]. Immunohistochemical staining of CEA in intestinal tissues has shown that in normal mucosa, CEA is located predominately at the apical, but possibly also at the basolateral membrane of the enterocytes. These findings are different in IBD patients. There is loss of staining in the basolateral membrane in ulcerative colitis and reduction of both api-

cal and basolateral staining in patients with Crohn's disease^[13]. CEA is attached to the outer leaflet of the plasma membrane by a glycosylphosphatidylinositol (GPI) anchor. GPI anchored proteins have been shown to use the GPI anchors as a sorting determinant for apical delivery^[14]. They are sorted by a lipid raft dependent mechanism. Depletion of cholesterol from Madin-Darby canine kidney cells (MDCK cells) results in misrouting of the proteins to the basolateral compartment^[15,16].

The putative activity of CEA at the apical and basolateral membranes prompted us to examine the impact of sorting of CEA in a polarized colonic cell line. This maneuver would shift its function from a molecule that binds and possibly promotes bacterial infection and inflammation to a molecule that may activate suppressor T cells and reduce the inflammatory response. In the present study, we were able to show that sorting of CEA to the apical and basolateral membranes can be altered by modifying the cholesterol content of the cell.

MATERIALS AND METHODS

Cells

Caco-2 cells were gifted by Professor Fricker, Institute for Molecular Biotechnology, University of Heidelberg. Caco-2 cells were cultured at 37°C in DMEM, supplemented with 10% FCS, 1% non essential amino acid solution, 1% sodium pyruvate, 100 U/mL penicillin, 100 µg/mL streptomycin and 2 mmol/L L-glutamine (Invitrogen, USA) and grown to achieve polarization for 14 d on 2.5 cm, 0.4 µm pore size Transwell polycarbonate filters (Costar, Cambridge, USA). The formation of an intact, confluent monolayer was monitored by measuring the transepithelial electrical resistance (TEER) and in representative experiments by detecting the unidirectional flux of inulin. Cell monolayers in Transwells were incubated in the presence of 3.6 mmol/L [3H]-inulin (NEN) in the basal well and 10 mmol/L methyl-β-cyclodextrin (MβCD, Sigma, USA) for 30-60 min. Two hours after MβCD treatment, radioactivity of the apical medium and of the basal medium were measured as described below using a Beckman LS6000 Scintillation Counter (Beckman, USA). The flux into the apical well was calculated as the percent of total inulin administered into the basal well. The same quantity of cells were used for the corresponding experiments.

Cholesterol depletion, pulse/chase experiments

For the cholesterol depletion study, the cells were grown for 1 d in either DMEM supplemented with 2 mmol/L L-glutamine, 10% FCS, 2 µmol/L lovastatin (Calbiochem, USA), and 0.25 mmol/L mevalonate or in complete medium. The following day, the cells were treated for 30-60 min with 10 mmol/L MβCD in methionine-free medium (labeling medium), and subsequently metabolically labeled with 100 µCi/dish of [35S]-methionine (NEN). The cells were chased for 2 h in labeling medium containing an excess of 150 µg/mL methionine and 20 µg/mL cycloheximide in order to inhibit protein synthesis. Cholesterol determinations were performed using the Amplex Red Cholesterol Assay kit (Molecular Probes). Proteins from the apical and basolateral medium were precipitated with

trichloroacetic acid and radioactivity from the precipitate was quantified in 5 mL of Ultima GoldTM liquid scintillation fluid (Packard Bioscience) using a Beckman LS6000 Scintillation Counter (Beckman Instruments).

Western blot analysis, CEA quantification

Media from the apical and basolateral chamber were separated on 10% polyacrylamide gel^[17], and proteins were transferred to a nitrocellulose membrane. The blot was probed with polyclonal CEA antibody (1:300, A0115, Dako, USA), and protein visualized using an HRP-conjugated secondary antibody and an enhanced chemiluminescence detection kit (Amersham, USA). CEA was quantified using an electrochemoluminescence immunoassay (ECLIA) from Roche Diagnostics that specifically recognizes CEA and not other CEA-like proteins.

Immunofluorescence

Filter grown Caco-2 cells were treated with lovastatin/mevalonate and MβCD as described above. The cells were fixed for 10 min with 4% PFA at 8°C followed by incubation with 0.1% Triton X-100. The fixed cells were incubated for 1 h at room temperature with the polyclonal antibody against CEA (1:200, Dako) or the monoclonal antibody against Na⁺/K⁺-ATPase (1:100, Affinity BioReagents, Golden, USA) in PBS containing 0.2% gelatine. After three washes with PBS containing 0.2% gelatine, the cells were incubated with the respective Cy3- or Cy2-labeled secondary antibodies (1:600 and 1:100, respectively) in PBS containing 0.2% gelatine for 1 h at room temperature. Confocal image acquisition were done on a Leica TCS SP2 system and pictures arranged with Adobe Photoshop.

Preparation of detergent resistant membranes (DRMs)

Detergent extraction with Triton X-100 was performed as described^[18,19]. The cells were grown on filters, washed once with PBS and scraped on ice into 300 µL 25 mmol/L Tris-HCl, pH 7.4, 150 mmol/L NaCl, 3 mmol/L EDTA (TNE) buffer containing 25 g/mL each of chymostatin, leupeptin, antipain and pepstatin A. The cells were homogenized through a 25 G needle and centrifuged for 5 min at 3000 r/min. The postnuclear supernatant was subjected to extraction for 30 min at 4°C in 2% Triton X-100/TNE. The extracts were adjusted to 40% OptiPrep (Nycomed, Oslo) and overlaid in a TLS 55 centrifugation tube with 30% OptiPrep/TNE, and TNE. After centrifugation for 2 h at 55000 r/min, six fractions were collected from the top, and Western blots were performed.

Statistical analysis

All values are reported as mean and standard error of the mean (mean ± SE). The Kruskal-Wallis test was used to test for statistical significance. Probability values of $P < 0.05$ were set as threshold for statistical significance.

RESULTS

Depletion of cholesterol from polarized Caco-2 cells

Cholesterol depletion was achieved by a combination of lovastatin/mevalonate treatment and MβCD extraction^[19]. Lovastatin decreases the de-novo synthesis of cholesterol

in the ER by inhibiting HMG-CoA reductase. Mevalonate is used to allow the synthesis of nonsterol products from mevalonate. This is necessary to reduce the toxicity of lovastatin treatment given alone^[16,20]. M β CD has been shown to selectively extract cholesterol from the plasma membrane, in preference to other lipids^[21]. Caco-2 cells were grown for 24 h in the presence of lovastatin/mevalonate, and immediately prior to metabolic labeling were treated with 10 mmol/L M β CD for a maximum 60 min. The extent of cholesterol extraction was monitored using the fluorimetric test. Lovastatin treatment alone resulted in about 10% reduction. The addition of M β CD reduced the total cellular cholesterol levels to about 43% of the levels in the control cells (Figure 1A). Increasing the time of exposure or the concentration of M β CD, and prolonged treatment with lovastatin had a deleterious affect on cell viability.

The following experiments were performed 30 min after treatment with 10 mmol/L M β CD. In order to determine that the cholesterol depleted Caco-2 cells were still fully polarized, the permeability of the commonly used paracellular marker [3H]-inulin was analyzed^[22]. Only small amounts of [3H]-inulin are able to move across polarized monolayers unless the tight junctions are altered or cell injury occurs^[23]. In representative experiments, no increased translocation from basal to apical location was observed after cholesterol depletion (Figure 1B). TEER was measured before and after cholesterol removal. In representative experiments again, there was no significant difference between cholesterol depleted and non-depleted cells ($99.0 \pm 8.1 \Omega/\text{cm}^2$ vs $106.9 \pm 9.8 \Omega/\text{cm}^2$; mean and SEM of 3 representative experiments). In addition, polarization was controlled by immunofluorescence of filter grown Caco-2 cells, analyzing the basolateral distribution of Na⁺/K⁺-ATPase. The distribution of this protein did not change after cholesterol extraction.

Effect of cholesterol on the overall transport capacity of newly synthesized proteins in the direction of the apical plasma membrane

To obtain a quantitative assessment of the effect of cholesterol depletion on apical and basolateral protein secretion and to analyze the role of cholesterol in the sorting of newly synthesized proteins in general, pulse/chase experiments were performed. Caco-2 cells were metabolically labeled for 1 h and the total counts of labeled proteins secreted within 2 h into the apical and basolateral medium were analyzed. The chase period was performed in the presence of cycloheximide to inhibit further protein synthesis. In non-depleted cells, the total secreted radioactivity in the apical chamber was $11.1\% \pm 3.4\%$. After cholesterol depletion, apical secretion was reduced to $4.7\% \pm 1.1\%$. This change was not due to a decrease in protein secretion in general. Whereas cholesterol depletion reduced apical secretion by 35%, at the same time, the basolateral secretion increased by 39% (Figure 2).

Effect of cholesterol depletion on polarized CEA secretion.

Under normal cell culture conditions, CEA family proteins are sorted predominately to the apical membrane. In polarized Caco-2 cells, there was no significant staining of

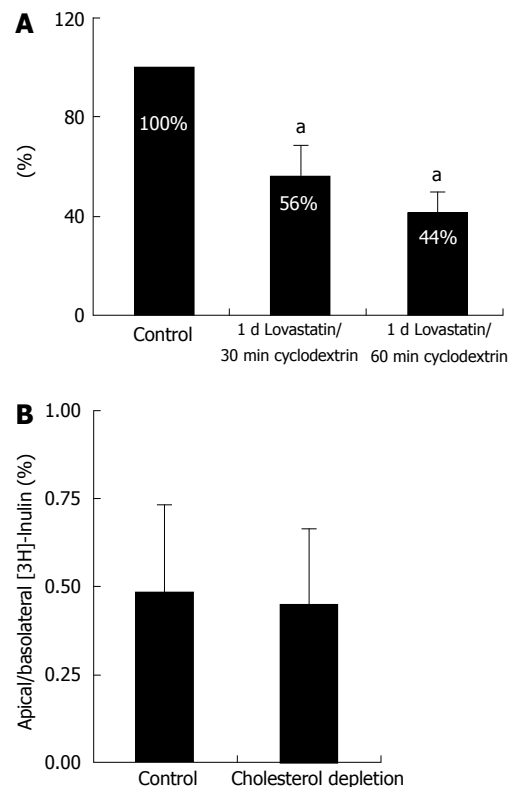


Figure 1 Cholesterol can be depleted from Caco-2 cells by a combination of lovastatin and M β CD. Cells were grown for 1d in the presence of lovastatin/mevalonate and then treated for 30-60 min with 10 mmol/L M β CD. **A:** Depending on the time of M β CD extraction, the total cellular cholesterol levels could be reduced by about 60%. Exposure for 30min with 10 mmol/L M β CD (2nd bar) was used for the following experiments. The cholesterol level was arbitrarily set to 100% in control cells. An asterisk indicates significant differences ($56.3\% \pm 17.1\%$ vs 100% and $46.3\% \pm 17.1\%$ vs 100%, both $^aP < 0.05$); **B:** [3H]-inulin permeability did not change after cholesterol depletion ($P > 0.05$).

CEA at the basolateral membrane (Figure 3A). In addition, no significant basolateral secretion of CEA could be measured by Western blot analyses. However, in cholesterol depleted cells, there was significant secretion of CEA from the basolateral membrane (Figure 3B). Immunofluorescent analyses of CEA in cholesterol depleted cells revealed still an apical staining of CEA, however the fluorescent band was much weaker than in the control cells, and no basolateral staining was detected. We believe that cleavage of CEA at the basolateral side may explain the difference between the staining and the amount of CEA present in the basolateral medium. Quantification of secreted CEA within 2 h after cholesterol depletion using a CEA specific electrochemoluminescence immunoassay revealed that apical secretion was reduced by $39\% \pm 15\%$ (Figure 3C). In non-depleted cells, no significant secretion of CEA was detected in the basolateral medium after 2 h (i.e., still below the detection level), whereas in depleted cells a concentration of $0.26 \pm 0.06 \mu\text{g/L}$ was obtained.

Effect of cholesterol depletion on association of CEA to DRMs

One way to analyze if a protein is found is to isolate DRMs. Association of a protein with DRMs is shown by its insolubility in detergents such as Triton X-100^[24], which leads to floatation to low densities in sucrose or OptiPrep

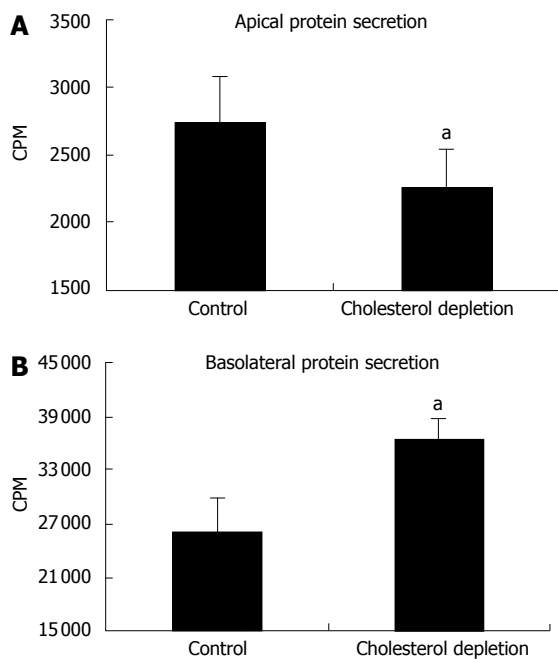


Figure 2 Effect of cholesterol depletion on polarized protein secretion. Filter-grown Caco-2 cells were pulsed for 1 h with [35 S]-methionine and the secretion of labeled proteins within 2 h was quantified using a scintillation counter. The total radioactivity of protein precipitates was (A) reduced in the apical medium of cholesterol depleted cells and (B) increased in the basolateral medium. An asterisk indicates significant differences (2736.6 ± 352.7 vs 2257.6 ± 282.4 and 28893.2 ± 3523.1 vs 38256.0 ± 2555.3 , $^aP < 0.05$).

gradients. After isolation of cellular membranes our experiments revealed that CEA is found in two membrane compartments within DRMs as well as in detergent soluble parts. Cholesterol depletion reduced significantly the association of CEA with DRMs (Figure 4).

DISCUSSION

The present data shows that the transport and secretion of CEA to either the apical or the basolateral membrane of polarized Caco-2 cells can be influenced by changing the cholesterol level of the cells. Under normal cell culture conditions, CEA is predominantly sorted and partially released at the apical membrane. Cholesterol depletion results in a significant shift to basolateral secretion.

These findings are in line with previous reports by Keller *et al* and Prydz *et al* who used the model of polarized MDCK^[15,16]. These workers showed that the overall transport capacity in the direction of the apical membrane is reduced and that the sorting of proteins with a high affinity for lipid rafts is influenced by cholesterol depletion. Therefore, these workers suggested the presence of two constitutive exit routes from the TGN towards the plasma membrane in polarized cells: An apical route, which is lipid raft-dependent, and a raft-independent basolateral route^[15,16]. Lipid rafts are small platforms, composed of sphingolipids and cholesterol in the outer exoplasmic leaflet, connected to phospholipids and cholesterol in the inner cytoplasmic leaflet of the cellular membranes. In mammalian cells, these are first assembled at the Golgi complex. From there, they move to the plasma membrane and spread into the endocytic pathway. Cholesterol is

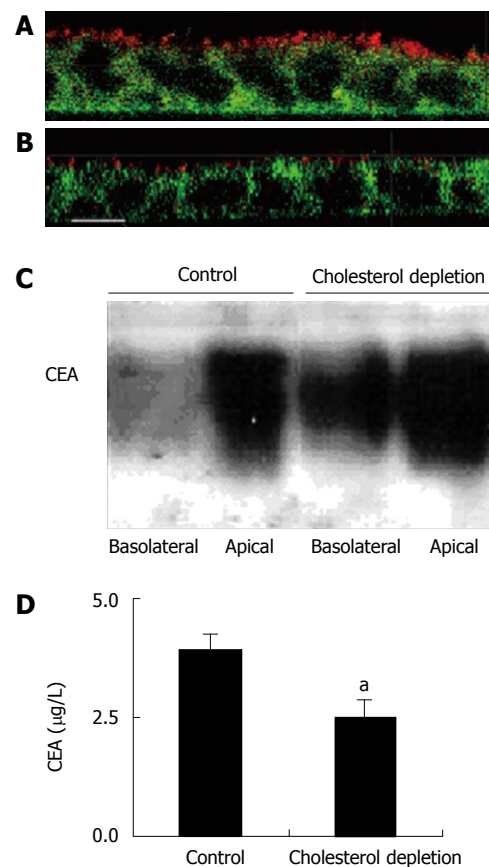


Figure 3 The effect of cholesterol depletion on apical and basolateral secretion of CEA. A, B: X-Z confocal views of cells labeled with antibodies directed against CEA (red) and Na⁺/K⁺-ATPase (green). In non-depleted cells under steady state conditions, CEA family proteins are expressed at the apical surface (A). Caco-2 cells were grown to confluency on polycarbonate filters for 14 d and cholesterol-depleted with a combination of lovastatin/MβCD. After cholesterol depletion, CEA was still apical but with a lower expression level (B) but no basolateral staining of CEA was detected (Bar 10 µm). C: Media from apical and basolateral chamber were collected for 2 h and Western blots performed. In non-depleted cells, most of the CEA is secreted into the apical medium. In cholesterol depleted cells, a significant amount was also found in the basolateral medium. D: Quantification of apical CEA secretion using an ECLISA from Roche. Six similar experiments were performed. An asterisk indicates significant differences (3.12 ± 0.62 vs 1.91 ± 0.81 , $^aP < 0.05$).

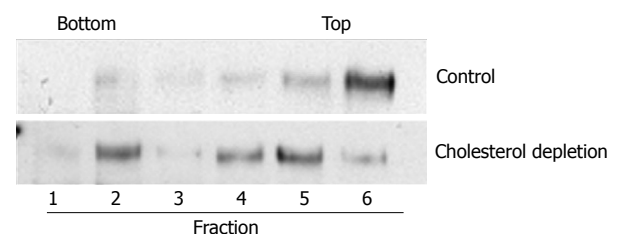


Figure 4 Cholesterol depletion reduces association of CEA with DRMs. Filter grown Caco-2 cells with or without treatment with a combination of lovastatin/MβCD were extracted on ice with 2% Triton X-100. After flotation in an OptiPrep step-gradient, fractions were collected and Western blots performed. Cholesterol depletion shifted CEA from the top (raft fractions) to the bottom fractions (non raft fractions).

believed to serve as a spacer between the hydrocarbon chains of the sphingolipids and to function as a dynamic glue that keeps the raft assembly together. Removal of raft cholesterol, leads to the disassociation of lipid rafts and affects its putative functions^[24]. Under normal cell culture

conditions, the majority of raft proteins are found at the apical membrane where in the presence of cholesterol depletion, these proteins are missorted to the basolateral membrane^[16]. Typical raft proteins are doubly acylated such as tyrosine kinases of the Src family, cholesterol-linked and palmitate-anchored proteins like hedgehog and GPI-anchored proteins to which CEA belongs^[24]. Therefore, under normal cell culture conditions, most of the CEA is sorted to the apical membrane and secreted into the apical medium (Figure 3). This picture changes following cholesterol depletion. CEA is sorted and secreted in the basolateral membrane, suggesting that it is no longer associated with lipid rafts. Our results on DRM association support this hypothesis (Figure 4). However, lowering the cholesterol levels is not only a specific treatment to influence lipid raft function, but it also has an impact on several other cellular processes such as lipid metabolism, endocytosis and enzyme activity^[25,26]. CEA release from the membrane is dependent upon the action of phospholipase D. It has been shown recently that its activity is increased after removal of cholesterol^[27]. How this phenomenon impacts the polarized secretion of CEA under low cholesterol conditions needs further evaluation.

Influencing the sorting of CEA may be relevant, since the compartmentation of the protein may have distinct functions. Whereas its apical expression may serve to bind mucus and microorganisms^[6-9], its basolateral expression influences the activation of anti-inflammatory suppressor T-cells that is important in controlling the inflammatory response of the gut^[28]. Therefore, an increased basolateral expression of CEA may be beneficial in the clinical course of IBD. IBD is thought to be the result of an uncontrolled inflammatory response. While the exact pathogenesis of the disease is not understood, it is likely that both the immune system as well as luminal agents (e.g. ingested nutrients or microbial agents) are important. Therefore, factors that are critical in permitting signals from the lumen to the mucosal immune systems may be important targets for therapy. It has been shown that intestinal epithelial cells from patients with IBD have reduced ability to activate suppressor T-cells and this correlates with the level of expression of CEA^[13]. It is interesting to note that whereas in normal intestinal tissue faint basolateral staining of CEA is detected, in patients with ulcerative colitis the basolateral staining is lost^[13]. Therefore, it is intriguing to speculate that cholesterol depletion of enterocytes in these patients may enhance the basolateral expression and therefore increase the reduced ability to activate CD8+ T-cells.

One therapeutic approach that has already been used in clinical studies in IBD is to alter the lipid composition of the cellular membranes. Lipid-based therapies such as the use of fish oil and phospholipids have been found to be successful^[29,30]. In addition, animal experiments employing ganglioside enriched diets and short chain fatty acids have shown reduction in intestinal inflammation^[31,32]. Interestingly, reducing cholesterol by systemic inhibition of HMG-CoA with pravastatin has also been found to reduce inflammation of DDS-induced colitis in rats^[33]. The authors noted that the effect of pravastatin was due to a decrease in the activity and expression of endothelial nitric-

oxide synthase (eNOS). Emerging data suggests, that even small changes in cellular cholesterol levels is successful in influencing lipid raft processes^[24,34]. However, whether different compartmentation of apical and basolateral expressed proteins may, in part, have contributed to the positive effect of statins, remains speculative. Therefore, whether the lipid based therapeutic approach in IBD should include reduction in the cholesterol content of enterocytes, remains an open question. Further studies are needed to determine the clinical significance of our findings.

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COMMENTS

Background

Carcinoembryonic antigen (CEA) is a GPI-anchored glycoprotein that is believed to have distinct functions depending upon its expression at the apical or basolateral plasma membrane. Whereas it is possible to interact with bacteria with the apical location, basolateral expression may be involved in the activation of suppressor T-cells and thus have an anti-inflammatory effect. A reduced expression at the basolateral compartment has been implicated in the pathogenesis of inflammatory bowel disease (IBD). Thus influencing the apical to basolateral transport ratio may be a potential tool in the treatment of IBD.

Research frontiers

Sorting of proteins to the apical or basolateral membrane is a precisely regulated mechanism. One part of this sorting is regulated by the dynamic interaction of protein with lipid rafts. Influencing lipid raft dependent mechanisms by changing the lipid content of cells may provide a new therapeutic approach to human diseases.

Innovations and breakthroughs

Apical sorting of CEA is cholesterol and presumably lipid raft dependent. After cholesterol depletion, basolateral sorting is increased, thus increasing the presumed basolateral anti-inflammatory properties. These results are in line with previous reports on apical sorting of lipid raft associated proteins.

Applications

Local depletion of cholesterol from the luminal side of the gut may be a therapeutic approach for inflammatory intestinal disorders. This can possibly be integrated in a lipid based approach in the treatment of IBD, as previously shown with the use of phospholipids and omega-3 fatty acids.

Peer review

The manuscript is very innovative. Changing the intracellular transport of a protein towards an anti-inflammatory route is interesting. However, the clinical relevance remains unclear.

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RAPID COMMUNICATION

Clinical predictors of colorectal polyps and carcinoma in a low prevalence region: Results of a colonoscopy based study

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Abstract

AIM: To estimate the prevalence of colorectal cancer (CRC) in patients with long lasting colonic symptoms undergoing total colonoscopy; and to establish clinical features predicting its occurrence.

METHODS: This prospective study was carried out in Imam Hospital, Tabriz University of medical sciences, Iran. Continuous patients with long lasting lower gastrointestinal tract symptoms who had the criteria of a colonoscopy were included. The endoscopist visualized the caecum documented by a photo and/or a specimen from terminal ileum.

RESULTS: Four hundred and eighty consecutive symptomatic patients [mean age (SD): 42.73 (16.21)] were included. The prevalence of colorectal neoplasia was 15.3% (34 subjects) and 37.7% (181 subjects) had a completely normal colon. Adenomatous polyps were detected in 56 (11.7%) patients, in 12.3% of men and 10.9% of women. The mean age of the patients with a polyp was significantly higher than the others (49.53 ± 14.16 vs 41.85 ± 16.26 , $P = 0.001$). Most of the adenomatous polyps were left sided and tubular; only 22.5% of polyps were more than 10 mm. Cancer was detected in 16 (3.6%) of our study population, which was mostly right sided (57.2%). The mean age of patients with cancer was significantly higher than the others (60.25 ± 8.26 vs 42.13 ± 16.08 , $P < 0.005$) and higher than patients with polyps [60.25 (8.26) vs 49.53 (1.91) ($P < 0.0005$)]. None of the symptoms (diarrhea, abdominal pain, rectal bleeding, constipation, altering diarrhea and constipation, history of cancer, known irritable bowel disease, history of polyp and fissure or family history of cancer) were predictors for cancer or polyps, but the age of the patient and unexplained

anemia independently predicted cancer.

CONCLUSION: Less advanced patterns and smaller sizes of adenomas in Iran is compatible with other data from Asia and the Middle East, but in contrast to western countries. Prevalence of colonic neoplasia in our community seems to be lower than that in western population. Colonic symptoms are not predictors for polyps or cancer but unexplained anemia and elder age can predict CRC.

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Key words: Colorectal cancer; Adenomatous polyp; Colonic symptom; Prevalence; Iran

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INTRODUCTION

Colorectal cancer (CRC) is a common cancer especially in industrial countries^[1]. The disease is related to a high mortality, morbidity and cost. It usually arises over several years from adenomas that undergo a series of specific genetic perturbations^[1] and therefore is a preventable disease when diagnosed early^[2] and treated.

Epidemiological studies of different nations and migrated populations strongly support the role of environmental factors as a reason for colorectal cancer such as dietary regimen^[3]. Its incidence is reported to be 35%-60% in US^[4] which is very close to European countries^[5].

It has been considered to be less common among Asians and Caucasians^[6,7]. However, recent studies have shown an increasing trend in these populations^[8-10].

Variation in the incidence of colorectal neoplasia has been observed in populations and even in cities of one country. In 1997 autopsy studies from Iran have reported the incidence of colorectal polyps to be 1.6%^[11]. Recent

increases in the incidence of CRC have been reported from cancer registry centers in our country as well^[12], but there is a lack of published prospective studies about endoscopic evaluation of CRC epidemiology in Iran. Therefore, we felt a genuine need to understand and to define better the characteristics of the in our local population; while our clinical evidence supports lower incidence of CRC compared to western countries.

Screening asymptomatic patients is the most common manner of detecting colorectal polyps in western countries, while most CRC cases are still detected once evaluating clinical symptoms^[13]. However most asymptomatic people do not accept a colonoscopy yet.

To find out epidemiological variations between Iranians compared with western populations, we carried out a study in symptomatic patients undergoing colonoscopy; investigating whether their symptoms were related to the chance of having a polyp or cancer.

MATERIALS AND METHODS

This prospective study was carried out in Imam Hospital, Tabriz University of medical sciences, Iran. Four hundred and eighty consecutive participants who visited the same gastroenterologist (17 years experience) because of unexplained lower gastrointestinal tract symptoms for more than 3 mo and underwent total colonoscopy between May 2005 and April 2007 were studied.

The endoscopist visualized the caecum in all subjects, documented by a photo of caecum and/or specimen of terminal ileum. Withdrawal time was 10-20 min. There was no significant morbidity associated with colonoscopy. A few patients were excluded as a result of failure to reach the caecum (because of poor preparation or technical problems) or because they were referred for polypectomy. A biopsy was performed in patients with an infiltrative lesion and/or polyp smaller than 5 mm, while polypectomy was done in polyps more than 1 mm. Polypectomy in 6-9 mm pedunculated polyps and multiple biopsies in sessile ones was achieved. Specimens were then histopathologically evaluated by a pathologist expert in GI disorders.

Data analysis was performed using SPSS version 13 software. The χ^2 test and student's *t* were used to determine the significance of associations between different symptoms and colonoscopic findings. Predictive factors for CRC including gender, age groups and symptoms were calculated using multiple logistic regression analysis. A two-tailed test was used for all and a *P*-value of < 0.05 was considered significant.

RESULTS

The 480 participants included 269 men (56%) and 211 women (44%). Mean age \pm SD of them was 42.73 ± 16.21 . The indications for colonoscopy are summarized in Table 1.

Taking adenoma and carcinoma together, 72 subjects (15.3%) were found to have colorectal neoplasia confirmed by histopathological examination of biopsy samples and

Table 1 Long lasting symptoms indicating a total colonoscopy and results regarding each symptom *n* (%)

Indicating symptom	Polyp	Cancer
Rectal bleeding (<i>n</i> = 142)	20 (14.1)	4 (2.8)
Diarrhea (<i>n</i> = 164)	7 (4.3)	1 (0.6)
Constipation (<i>n</i> = 48)	3 (6.3)	2 (4.2)
Altering bowel habit (<i>n</i> = 27)	1 (3.7)	1 (3.7)
Abdominal pain (<i>n</i> = 147)	16 (10.9)	7 (4.8)
Irritable bowel disease (<i>n</i> = 7)	1 (14.3)	0
History of polyp (<i>n</i> = 4)	0	0
Unexplained anemia (<i>n</i> = 35)	7 (20.0)	5 (14.3)
Cancer in other organs (<i>n</i> = 25)	4 (16.0)	2 (8.0)
Family history of cancer (<i>n</i> = 10)	2 (20.0)	0
Abnormal barium enema (<i>n</i> = 15)	3 (20.0)	0
Others (<i>n</i> = 8)	1 (12.5)	1 (12.5)

181 (37.7%) had a completely normal colon. Inflammatory bowel disease was diagnosed in 56 (11.7%), hemorrhoid in 120 (25%), fissure in 14 (2.8%), lipoma in 3 (0.6%), erosion or inflammation in 27 (5.6%). Other findings included fistula, angiodysplasia and diverticle.

During the study we found eight (1.6%) hyperplastic polyps. Four (25%) patients with CRC had synchronous polyps. Adenomatous polyps were detected in 56 patients (11.7%) including 12.3% of men and 10.9% of women ($P = 0.643$); 8 patients (14.3%) had multiple polyps. The mean age of patients with a polyp was significantly higher than that of the others (49.53 ± 14.16 vs 41.85 ± 16.26 $P = 0.001$). The distribution of adenomatous polyp and cancer according to age trend is described in Table 2. The mean time of symptoms in patients with a polyp (26.58 mo) was not significantly different compared with those without a polyp (27.38 mo).

The mean age of patients and polyp sizes in relation to polyp location are shown in Table 3. The location of the polyps was caecum (4.0%), ascending colon (10.0%), transverse colon (14.0%), descending colon (26.0%), sigmoid (26.0%) and rectum (20.0%). Most of the polyps were tubular (65.0%) followed by tubulo-villous (25.0 %) and villous adenomas (10.0%).

Cancer was detected in 16 (3.6%) which included 3.3% of men and 3.3% of women ($P = 0.986$). The mean age \pm SD of patients with cancer was significantly higher than that of patients without cancer (60.25 ± 8.26 vs 42.13 ± 16.08 $P < 0.005$) and that of patients with polyps (49.53 ± 1.91 , $P < 0.0005$). The mean duration of symptoms was significantly shorter compared with patients without cancer (6.88 mo in patients with cancer vs 27.81 in patients without, $P < 0.005$). The location of the cancer was caecum (14.3%), ascending colon (42.9%), transverse colon (7.1%), sigmoid (14.3%) and rectum (21.4%).

Associations between any of these symptoms and the findings on colonoscopy was assessed by a logistic regression model. Unexplained anemia was an independent predictor for cancer ($P = 0.004$). Unexplained anemia kept its predictive value even after adding age, gender and the duration of the symptoms in the analysis ($P = 0.006$), while age was found as an independent predictor of cancer as well ($P = 0.037$).

Table 2 Age distribution of colorectal neoplasm detected by total colonoscopy in Iranian symptomatic patients *n* (%)

	< 30 (<i>n</i> = 108)	30-39 (<i>n</i> = 110)	40-49 (<i>n</i> = 90)	50-59 (<i>n</i> = 88)	60-69 (<i>n</i> = 48)	70-79 (<i>n</i> = 31)	80 < (<i>n</i> = 3)	All (<i>n</i> = 480)
Colorectal cancer	0	0	2 (2.2)	6 (6.8)	4 (8.3)	4 (12.9)	0	16 (3.6)
Colorectal adenomatous polyp	5 (4.6)	10 (9.1)	11 (12.2)	18 (20.5)	7 (14.6)	5 (16.1)	0	56 (11.7)

Values in parentheses are percentages.

Table 3 Age and size of the polyp in the patients according to polyp location (mean \pm SD)

	Size (mm)	Age (yr)
Rectum	8.83 \pm 13.22	41.80 \pm 13.83
Sigmoid	13.00 \pm 8.48	57.33 \pm 8.43
Descending colon	10.18 \pm 9.08	49.69 \pm 12.10
Transverse colon	5.16 \pm 3.18	41.29 \pm 20.05
Ascending colon	6.60 \pm 1.51	59.20 \pm 8.28
Caecum	8	51.50 \pm 7.77

Patients with diarrhea and constipation had lower chances of having a polyp ($P < 0.0005$ and $P = 0.025$ receptively). Diarrhea kept the negative predictive value even after adding age, gender and duration of the symptoms in analysis ($P = 0.022$) while age was found as an independent predictor of polyps ($P = 0.006$).

There was no significant relation between age and location of the polyp or cancer ($P = 0.606$ and $P = 0.283$). The location of the cancer or the polyp was not related to the gender of the patients either ($P = 0.336$ and $P = 0.256$).

DISCUSSION

This report represents the first study to characterize the profile of CRC in symptomatic patients of Iran. Although this is not a population based study, important data about this cancer in a sample of our local population are presented.

The only published report from our province shows age-adjusted rates of CRC (as the 3rd most common GI cancer) to be 6.7 in males and 5.2/100 000 in females in our region^[14], which may be influenced by the pathology based manner of the study in part. A report of a recent population based registry from five provinces of Iran showed age-adjusted rates of CRC in Iranian males and females to be 8.2 and 7.0/100 000, respectively while 17% of the cases were younger than 40 years of age at the time of diagnosis^[15]. This rate decreased to 2%-8% in western countries^[16]. In contrast to the low risk of CRC in our country, research supports an increase in occurrence of the disease^[17]. This increase in incidence of CRC in the Asia-Pacific region has been related to the dramatic socio-economic developments^[18] which can in part explain the higher rate in the younger aged population. The use of fat and thereby prevalence of obesity have increased during the past decade^[19,20]. Appropriate screening strategies should be considered to decrease the burden of CRC in the young population of Iran. The first step is gathering

satisfactory data on characteristics of the disease to map out an effective plan.

Colorectal adenoma and CRC were detected in 11.7% and 3.6% of our study population with long lasting colonic symptoms. This low rate is compatible with other published data from Asia and Middle East^[15]. Colonoscopic examination from western countries has shown neoplastic lesions in 37.5% of asymptomatic patients^[21] which is even higher than our symptomatic (including evidence of bleeding) sample. Genetic factors and the high fibre diets of our community may explain this low incidence but this can be influenced by recent changes like westernization of diet and reduction in physical activity of our population.

No gender differences were noted in our patients. The mean age of diagnosis in our cancer patients and patients with adenomatous polyps (in the 5th decade of life) is consistent with previously established data. Our cancer patients were more than 10 years older than patients with adenomatous polyps which is compatible with transformation of a polyp to cancer in a 10-year period. The incidence of colorectal cancer increases with age. Risk of colorectal cancer increases slightly after the age of 40 and more sharply after 50 years^[22]. This pattern was also observed in our study. Older age is an independent predictor for polyp and cancer in our population. Lower gastrointestinal symptoms are not predictive of colorectal neoplasia in our study as supported by reports from western countries^[23]. This may be explained simply by the frequency of lower gastrointestinal symptoms in the general population. However, the rate of rectal bleeding in this study is considerably higher than that in the general population, while a possible explanation is that there is a high prevalence of benign disease likely to cause rectal bleeding. Unexplained anemia is the only sign which can predict CRC.

The characteristics of both patients and adenomas can influence the risk of carcinoma developing in colorectal adenomas. These are the age of the patient, size of the adenoma, its villous component and the severity of dysplasia^[24]. Villous adenomas seem to be less common in our community compared with western countries, which may become malignant in 29% to 70% of the time^[25-27]. The majority of polyps detected in our study population were tubular (65%) and villous adenomas comprised 10%.

The size of polyps has been reported as an independent predictor of malignancy in colorectal polyps^[28,29]. The risk of developing adenocarcinoma is 1% in adenomas of up to 1 cm in size, 10% in adenomas from 1 cm to 2 cm in diameter and 50% in those greater than 2 cm in diameter^[30]. Only 4% of adenomas less than 6 mm diameter, and 16% of those between 6 mm and 10 mm are reported to have

unfavorable histology^[31]. According to our results, 40.0% of polyps were less than 6 mm, 37.5% were between 6-10 mm and 22.5% were more than 10 mm which shows smaller groups at higher risk of malignancy.

There has been much discussion about the anatomical distribution of colorectal tumours. A “left” to “right” sided or proximal shift of tumours has been reported in studies mostly from western countries^[32-35]. Nevertheless, several other studies especially from Asia have shown no such shift^[36-42]. In the authors’ experience in our region about anatomic sites of adenomatous polyps and CRC (1992 to 2005), there is a predominant location of left side^[43]. The study was based on evaluation by colonoscopy and barium enema in patients with colorectal adenoma or CRC. In the current study which is based on findings of total colonoscopy, more than 40% of polyps were located in the recto-sigmoid region while a shift to the right side was prominent in the sites of CRC. The former retrospective study focused on patients with adenomatous polyp or CRC among patients evaluated by colonoscopy. Based on this study with a large number of cases, an anatomical distribution pattern and left shift of colorectal adenomas and cancer is compatible with data from most Asian countries. However, the current study was designed on patients with different lower GI symptoms evaluated for colorectal neoplasms. The right side dominance of CRC in this study is more likely not due to a true increase in right-sided tumors but may be a result of a small number of recorded cancer patients as well as younger ages in our study population.

Some previous published studies showed older ages of patients with right-sided tumours. No difference in the age of diagnosis of right-sided and left-sided tumours was observed in our patients which has been noted in other studies as well^[7].

Although most cases of rectal bleeding were due to self limiting diseases in our study, the probability of colorectal cancer increases significantly both in people older than 60 years and in association with unexplained anemia indicating the need for a more thorough investigation in such cases.

Smaller size of adenomas, dominance of tubular type and fewer cases with severe dysplasia among colorectal adenoma and lower incidence of cancer in our region compared with western populations is comparable with Asian populations and may suggest that it is not a serious health problem of our community at present. Although suspicious symptoms clearly call for urgent investigation, it is important to recognize lower gastrointestinal symptoms are not predictive of cancer except for unexplained anemia in elderly patients.

COMMENTS

Background

Colorectal cancer (CRC) is a common cancer especially in industrial countries and is a preventable disease when diagnosed and treated early.

Research frontiers

Screening asymptomatic patients is the most common way of detecting colorectal polyps while most cancer cases are still detected once evaluating clinical

symptoms. However most asymptomatic people do not accept endoscopy yet. We carried out a study in symptomatic patients undergoing colonoscopy; investigating whether their symptoms were related to chance of having a polyp or cancer.

Related publications

Colonoscopic examination from western countries has shown neoplastic lesions in 37.5% of asymptomatic patients which is even higher than our symptomatic. An increase in the incidence of CRC in the Asia-Pacific region is noted and has been related to the dramatic socio-economic developments.

Innovations and breakthroughs

This report represents the first study to characterize the profile of CRC in symptomatic patients of Iran. Although this is not a population based study, important data about this cancer in a sample of our local population are presented.

Applications

Colonic symptoms are not a predictor for polyp or cancer but unexplained anemia and older age can predict CRC. Less advanced patterns and smaller sizes of adenomas in Iran is compatible with other data from Asia and Middle East, in contrast to western countries. The prevalence of colonic neoplasia in our community seems to be lower than that in western populations.

Terminology

Colorectal polyps arise from normal epithelium of the colon after some abnormal changes in genes and may develop to colorectal cancer over time.

Peer review

This is a potentially interesting manuscript, which describes a prospective colonoscopy based study investigating the prevalence of colorectal polyps and cancer in patients with persistent (> 3 mo) colonic symptoms with the aim of determining whether or not the indications were predictive for the presence of either polyp or CRC. The study group was large (480 patients), indications for colonoscopy diverse and the average age profile relatively young (about 43 years). The results section was rather disjointed and could have been better presented and aided by additional tables and figures.

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Gastric juice for the diagnosis of *H pylori* infection in patients on proton pump inhibitors

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Abstract

AIM: To determine the efficacy of gastric juice polymerase chain reaction (PCR) for the detection of *H pylori* infection in comparison with histology and gastric antral biopsy PCR in patients on a proton pump inhibitor (PPI).

METHODS: Eighty-five consecutive patients with dyspeptic symptoms were enrolled. Gastric biopsies for histology, PCR and gastric juice were collected at endoscopy for PCR of the *H pylori* urease C gene (ure C). Sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), accuracy, positive and negative likelihood ratio for PCR of gastric juice for the *H pylori* ure C gene was compared to histology and gastric antral biopsy *H pylori* ure C PCR in patients with and without PPI.

RESULTS: Gastric juice PCR was positive in 66 (78%) patients. Histology showed *H pylori* associated gastritis in 57 (67%). Gastric biopsy PCR was positive in 72 (85%). In patients not taking PPI, the sensitivity, specificity, PPV, NPV, accuracy and positive and negative likelihood ratio for gastric juice PCR were 89%, 72%, 91%, 67%, 90%, 85%, 3.1 and 0.1 respectively. In patients on PPI these values were 86%, 100%, 100%, 29%, 86%, 9.5 and 1.4, respectively.

CONCLUSION: Gastric juice PCR for the diagnosis of *H pylori* infection has increased sensitivity compared to histology with PPI. The use of gastric juice PCR is recommended to confirm *H pylori* status in patients taking PPIs.

INTRODUCTION

H pylori is a spiral gram negative microaerophilic bacterium that infects the human gastric mucosa and is associated with gastritis, gastroduodenal ulcer disease, gastric carcinoma and mucosal associated lymphoid tissue lymphoma^[1,2]. The prevalence of *H pylori* is high in developing countries. A recent study revealed an early colonization/infection of infants with *H pylori* and a prevalence of 67% at 9 mo of age in a peri-urban community in Karachi, Pakistan^[3]. *H pylori* serology was positive in 58% of our general population in a previous study but is likely to actually be higher^[4]. Currently, several diagnostic tests of varying sensitivity and specificity are available for determining the presence of *H pylori*, which include rapid urease test (RUT), histology, culture, urea breath test (UBT), and serology. Isolation of *H pylori* from gastric biopsy specimens constitutes the most specific way to establish the diagnosis of infection and to study the genotype of the infecting strains, however, it is a time consuming process. RUT and histology are still commonly used for the diagnosis of *H pylori* infection in our country as other modalities of *H pylori* testing, such as UBT and facilities for *H pylori* stool antigen tests, are not widely available.

In Pakistan, self-prescription is common and medications are available over the counter without prescriptions^[5]. It is known that acid reducing drugs; e.g. proton pump inhibitor (PPI), histamine-2 receptor blocker (H2RB), antibiotics and bismuth compounds reduce the sensitivity and specificity of the diagnostic tests for *H pylori*^[6,7]. In our previous studies, we demonstrated that histology is comparatively less affected by PPI than RUT^[8,9]. Polymerase chain reaction (PCR) is a highly

sensitive technique that can detect very small amounts of DNA. The DNA molecule is chemically stable and can survive in the environment for long periods^[10]. PCR may be useful, therefore, in detecting the presence of *H pylori*, even when the organism is in a nonculturable state. In previous studies, gastric juice PCR has been evaluated as a highly specific and rapid method for the detection of *H pylori*^[11,12]. An efficient and accurate diagnosis of *H pylori* infection is important when seeking to cure patients with persistent gastric symptoms in which *H pylori* infection is suspected. The aim of this study was to determine the efficiency of gastric juice PCR for the detection of *H pylori* infection in patients on PPI and compare it with histology and gastric biopsy PCR.

MATERIALS AND METHODS

Patients

Eighty-five consecutive patients with dyspeptic symptoms attending the gastroenterology outpatient clinic from February-November 2006 were enrolled. There were 58 (68%) males and 27 (32%) females. The age range was 17-70 years with a mean age of 36.8 ± 11 . Patients were divided into two groups: (1) those who received PPI (mainly omeprazole 20 mg once a day) for at least 4 wk before undergoing esophagogastroduodenoscopy (EGD); (2) patients with no previous treatment with antibiotics, PPI, H2RB and bismuth compounds. Patients in each respective group also did not use other drugs. Compliance with treatment was ascertained during an outpatient visit before the endoscopy. The study was approved by the Ethics Review Committee of Aga Khan University Hospital. Informed consent was obtained from all patients for EGD with biopsies from the antrum and corpus of the stomach and aspiration of gastric juice. EGDs were performed after an 8 h fast. A sample of gastric juice (5 mL) was aspirated at endoscopy by means of a sterile cannula used for endoscopic retrograde cholangiopancreatography (ERCP), passed through the suction channel and collected in a disposable sterile syringe. After each examination the endoscopes were washed with 2% glutaraldehyde and disinfected with 70% ethanol followed by rinsing with sterile water after each examination. Biopsy forceps were sterilized by autoclaving to ensure lack of cross contamination using the endoscopic equipment. All patients received conscious sedation with intravenous midazolam and topical pharyngeal anesthetic spray. Sterilized biopsy forceps were used to obtain gastric biopsy specimens from the antrum and mid of the corpus. Two biopsy specimens were removed from each site for histology and dispatched in a formalin containing container. Biopsy for PCR was dispatched in normal saline. Sensitivity, specificity, PPV, NPV, accuracy, positive and negative likelihood ratio for gastric juice *H pylori* ure C gene PCR were compared against histology and gastric biopsy PCR in patients with and without PPI to establish the efficiency of this diagnostic approach.

Histology

Gastric biopsy specimens from each site for histopathology

were stained with hematoxylin and eosin and Giemsa stain for the detection of *H pylori*; the degree of gastritis as determined by hematoxylin and eosin (HE) stain was scored in accordance with the Sydney system^[13].

Extraction of genomic DNA from gastric juice

Extraction of genomic DNA from gastric juice was carried out as previously described^[11]. A 5 mL of gastric juice aspirate was buffered to a neutral pH with 5 mL of Tris (0.67 mol/L, pH 7.4). Each sample was then concentrated by centrifugation at $10000 \times g$ for 20 min. The supernatants were removed and the pellets were resuspended in 100 μ L of sterile distilled water. One hundred μ L of lysis buffer [100 mmol/L NaCl, 10 mmol/L Tris-HCl (pH 8.0), 25 mmol/L EDTA, 0.5% sodium dodecyl sulfate], and 5 μ L of proteinase K (10 g/L) were added. Incubation was carried out at 50°C for 20 h; this was followed by phenol-chloroform extraction and ethanol precipitation. The resulting pellet was allowed to dissolve in 35 μ L of TE buffer 10 mmol/L Tris-HCl (pH 7.4) and 0.1 mmol/L EDTA (pH 8.0) for 20 h at 37°C. Samples were stored at -20°C before PCR amplification was performed. DNA content and purity was determined by measuring the absorbance at 260 nm and 280 nm using a spectrophotometer (Beckman DU-600, USA).

Extraction of DNA from gastric biopsy

Briefly, gastric tissue was homogenized to uniformity in 500 mL of sterile water and centrifuged at $12000 \times g$ for 3 min. Five hundred μ L of lysis buffer [100 mmol/L NaCl, 10 mmol/L Tris-HCl (pH 8.0), 25 mmol/L EDTA, 0.5% sodium dodecyl sulfate], and 10 μ L of proteinase K (10 g/L) were added. Incubation was carried out at 56°C for 20 h; this was followed by phenol-chloroform extraction and ethanol precipitation. The resulting pellet was allowed to dissolve in 40 μ L of TE buffer [10 mmol/L Tris-HCl (pH 7.4) and 0.1 mmol/L EDTA (pH 8.0)] for 20 h at 37°C. Samples were stored at -20°C before PCR amplification was performed. DNA content and purity was determined by measuring the absorbance at 260 nm and 280 nm using a spectrophotometer (Beckman DU-600, USA).

PCR for ure C

PCR was performed using extracted DNA as the template and urease gene C for primers. Forward primer (5'-TG GGACTGATGGCGTGAGGG-3') and reverse primer (5'-AAGGGCGTTT'TTAGATTTT-3') were prepared from the urease gene sequence according to the report of Labigne *et al*^[14]. PCR amplification was carried out in a total volume of 50 μ L containing 2 μ L of 2 mmol/L dNTPs, 1 μ L containing 50 pmol of primer 1, 1 μ L containing 50 pmol of primer 2 (synthesized by ABI Automatic synthesizer), 1 unit of Taq DNA polymerase (Promega), 5 μ L of $10 \times$ PCR reaction buffer, 3 mmol/L of MgCl₂, 2 μ L of DNA template containing 0.5 ng of extracted DNA and total volume rounded to 50 μ L by double distilled water. The reaction was carried out in a Perkin Elmer 9700 thermal cycler. The amplification cycle consisted of an initial denaturation of target DNA at 95°C for 5 min and then denaturation at 94°C for 1 min, primer

Table 1 Comparison of histology, gastric biopsy and juice PCR for the diagnosis of *H pylori* infection with and without PPI *n* (%)

	Medication		<i>P</i> value
	On PPI <i>n</i> = 37	Without PPI <i>n</i> = 48	
Histology			
<i>H pylori</i> positive gastritis	23 (62)	34 (71)	0.24
<i>H pylori</i> negative gastritis	14 (38)	14 (29)	
Gastric juice PCR			
Positive	30 (81)	36 (75)	0.50
Negative	7 (19)	12 (25)	
Gastric biopsy PCR			
Positive	35 (95)	37 (77)	0.02
Negative	2 (5)	11 (23)	

annealing at 56°C for 1 min and extension at 72°C for 1 min. The final cycle included an extension step for 5 min at 72°C to ensure full extension of the product. Samples were amplified through 35 consecutive cycles. Negative reagent control reactions were performed with each batch of amplifications, consisting of tubes containing distilled water in place of the DNA samples. Five µL of PCR product was electrophoresed on a 1.5% agarose gel to ensure homogeneity and yield. PCR amplification resulted in a homogeneous DNA fragment of the expected size of 820 bp for ure C gene.

Statistical analysis

The statistical package for social science SPSS (Release 11.5, standard version, copyright © SPSS; 1989-99) was used for data analysis. The descriptive analysis was done for demographic and clinical features. Results were presented as mean ± SD for quantitative variables and number (percentage) for qualitative variables. Odd ratio (OR) and 95% confidence interval (95% CI) were estimated to check the strength of association. Sensitivity, specificity, PPV, NPV, accuracy and negative and positive likelihood ratio were determined for PCR and histology.

RESULTS

Thirty seven (43.5%) patients were on PPI while 48 (56.5%) were not taking any medications. Abdominal pain was present in 60 (71%) and dyspepsia 25 (29%). Symptoms were equally common in two groups. The endoscopic diagnosis was pangastric erythema in 56 (66%), antral erythema in 25 (29.4%), gastric ulcer 1 (1.2%), gastric carcinoma 2 (4.2%), and duodenal ulcer 1 (1.2%). The age range of these patients was 25-70 years with mean age 43.5 ± 13.2.

Comparison of histology, gastric biopsy and juice PCR with and without PPI

Histology showed *H pylori* associated gastritis in 57 (67%) and *H pylori* negative gastritis in 28 (33%). On PPI, 23 (62%) had *H pylori* positive gastritis *P* = 0.24 (Table 1). Gastric juice ure C PCR was positive in 66 (78%) and negative in 19 (22%). On PPI, gastric juice PCR was positive in 30 (81%) *P* = 0.50 (Table 1). Gastric biopsy ure C PCR

Table 2 Comparison of gastric juice PCR and histology using gastric biopsy PCR as the gold standard *n* (%)

	PCR of gastric biopsy		<i>P</i> value
	Positive	Negative	
Over all (<i>n</i> = 85)			
Histology			
Positive	56 (78)	1 (8)	< 0.001
Negative	16 (22)	12 (92)	
Gastric juice PCR			
Positive	63 (87)	3 (23)	< 0.001
Negative	9 (13)	10 (77)	
On PPI (<i>n</i> = 37)			
Histology			
Positive	23 (66)	0	0.17
Negative	12 (34)	2 (100)	
Gastric juice PCR			
Positive	30 (86)	0	0.003
Negative	5 (14)	2 (100)	
No PPI (<i>n</i> = 48)			
Histology			
Positive	33 (89)	1 (9)	< 0.001
Negative	4 (11)	10 (91)	
Gastric juice PCR			
Positive	33 (89)	3 (27)	< 0.001
Negative	4 (11)	8 (73)	

was positive in 72 (85%) and negative in 13 (15%). On PPI, gastric biopsy PCR was positive in 35 (95%) with *P* = 0.02 (Table 1).

Comparison of histology and gastric juice PCR with gastric biopsy PCR

Seventy two (85%) were positive by ure C PCR of gastric biopsy compared to 57 (67%) by histology and 66 (78%) by gastric juice ure C PCR with *P* < 0.001 and *P* < 0.001, respectively (Table 2). For patients taking PPI, 23 (62%) were positive by histology while 30 (81%) were positive by gastric juice PCR with *P* = 0.17 and *P* = 0.003, respectively (Table 2). For patients not taking PPI, histology was positive in 34 (71%) while 36 (75%) were positive by gastric juice PCR with *P* < 0.001 and *P* < 0.001, respectively (Table 2). For patients taking PPI, the sensitivity, specificity, PPV, NPV, accuracy and positive and negative likelihood ratio for gastric juice PCR and histology were 86%, 100%, 100%, 29%, 86%, 9.5 and 1.4 and 73%, 100%, 100%, 14%, 0.6 and 2.7, respectively (Table 3).

DISCUSSION

Gastric juice represents a pooled source of events in the entire gastric microenvironment, and it may be valuable for studying *H pylori* whose mucosal distribution is patchy and variable. This is even more relevant in the setting of developing countries, where *H pylori* possibly exists as a dynamic mix of quasi-species. A single biopsy sample may not be able to detect the presence of *H pylori*, whereas gastric juice, being a more global sample, may overcome this limitation because the gastric juice reflects the actual microenvironment and the global level of infection in the stomach. *H pylori* has a very potent urease activity, and because of this highly specific activity of the urease enzyme *H pylori* are able to hydrolyze the urea present in

Table 3 Comparison of gastric juice PCR and histology with and without PPI using gastric biopsy PCR as the gold standard

	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Likelihood ratio LR +	Likelihood ratio LR-	Accuracy (%)
Over all (n = 85)							
Histology	77	92	98	43	9.6	0.25	80
Gastric juice PCR	87	77	95	53	3.7	0.16	85
On PPI (n = 37)							
Histology	73	100	100	14	0.6	2.7	74
Gastric juice PCR	86	100	100	29	9.5	1.4	86
No PPI (n = 48)							
Histology	89	90	97	71	0.89	0.12	85
Gastric juice PCR	89	72	91	67	3.17	0.15	85

the stomach. This serves to protect the organism from the harmful effects of gastric acid and the ammonia generated protects the organism by buffering gastric acid^[15,16]. The proton pump inhibitor reduces gastric acid secretion and inhibits urease activity^[17,18]. When the secretion of acid is suppressed, *H pylori* in the presence of urea increases the pH of its local environment to alkaline values and are unlikely to survive in a culturable form^[19].

This study has demonstrated that for patients taking PPI gastric juice *H pylori* PCR with a specific primer for ure C was more sensitive than histology to detect *H pylori* infection 86% vs 73% (Table 3). Gastric juice PCR was able to detect positively 7 (19%) patients who were negative on histology. This could be due to a patchy distribution of the *H pylori*, obtaining biopsies from an uninfected sites resulting in false negatives on histology and PPI activity against *H pylori*^[20]. The histology-negative, PCR-positive subjects were older, with a mean age of 43.5 ± 13.2 years. This is in keeping with a previous study^[21]. *H pylori* infection associated atrophy and intestinal metaplasia progresses with age^[22,23]. Thus, older patients may be more liable to have false-negative results from the commonly biopsied sites in our practice. Although, the overall sensitivity of the gastric juice PCR as seen in the present study is low. This might be attributed either to the lack of *H pylori* in the gastric juice or the presence of some inhibitor of PCR decreasing sensitivity of the technique. In this study, we did not choose to compare RUT with gastric juice PCR as it is already known that PPI reduces the sensitivity of RUT^[7-9]. In patients on PPI, the biopsy specimen may contain low bacterial density of viable cells giving a negative urease test. This will also lead to the lack of *H pylori* identification on histology. Similarly, culture of *H pylori* from gastric mucosal biopsies is likely to be negative with PPI as they are not only known to effect the distribution of *H pylori* within the stomach but are also detrimental to *H pylori* in both the antrum and the corpus^[7,24].

Proton pump inhibitors have been reported to modify the level of *H pylori* gastritis^[20,25,26]. The sensitivity, NPV, accuracy and positive likelihood ratio of gastric juice PCR was greater and the negative likelihood ratio was less for *H pylori* in patients on PPI, while specificity and PPV was similar to histology (Table 3). A previous study by Dickey *et al* showed histological examination sensitivity, specificity, PPV, NPV and diagnostic accuracy were reduced on acid reducing drugs^[7]. In their study, five (83%) of the histology-negative, seropositive patients taking PPI had

histological changes consistent with *H pylori* gastritis even though no *H pylori* were detected^[7]. In our study, the detection rate of *H pylori* was greater by gastric juice PCR on PPI (Tables 1-3). Of the various tests that are available for *H pylori* detection, histological examination of gastric biopsy is considered the most accurate method of diagnosis^[27]. If more than one gastric biopsy tissue is used it might improve the test sensitivity without compromising its specificity. Obtaining a serological test in these cases in our population, will not help in deciding whether to treat or not, as a positive result with serology does not tell whether the patient has a current infection or had a past infection that is now cured. The drawbacks of treating these patients who are not actively infected include among many others contribution to antibiotic resistance.

This is the first study to investigate influence of PPI on the results of PCR of gastric juice and histological examinations while using gastric biopsy PCR as the gold standard. In the presence of PPI, gastric juice PCR was more sensitive than histology. Seven (19%) subjects who were negative for *H pylori* by histology and positive by the gastric juice PCR assay should be regarded as having ongoing infections. These patients would have benefited from antimicrobial therapy. However, the results of this study needs to be confirmed in a larger group of patients. In conclusion, the use of the gastric juice PCR can be recommended to exclude *H pylori* infection in patients taking PPI. However, it can also be used as an additive test to confirm the *H pylori* status in patients having histological changes consistent with *H pylori* gastritis though negative for *H pylori*.

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COMMENTS

Background

An efficient and accurate diagnosis of *H pylori* infection is important when seeking to cure patients with persistent gastric symptoms in which *H pylori* infection is suspected. In patients on acid reducing drugs such as proton pump inhibitors (PPI), H-2 receptor blockers (H-2RB) *etc*, the accuracy of the rapid urease test, urea breath test and histology are known to be less accurate for the diagnosis of *H pylori* infection.

Research frontiers

The development of new types of test or targets to test for the *H pylori* infection in patients with prior use of PPI etc is important considering the morbidity and mortality associated with this infection.

Innovations and breakthroughs

This study determined the efficiency of gastric juice polymerase chain reaction (PCR) for the detection of *H pylori* infection in patients on PPI and compared it with histology and gastric biopsy PCR.

Applications

It showed gastric juice PCR for the diagnosis of *H pylori* infection had an increased sensitivity compared to histology in patients on PPI. The use of the gastric juice PCR can be recommended to confirm the *H pylori* status in patients taking PPIs.

Terminology

PPI: proton pump inhibitors are a group of drugs whose main action is pronounced and long-lasting reduction of gastric acid production. They are the most potent inhibitors of acid secretion available today.

Peer review

The paper means a real advance in the methodology of diagnosis in this field. The conclusions are valuable. The design is original. The methodology is correct and the results are well presented. Statistical analysis is adequate.

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RAPID COMMUNICATION

High rate of complicated idiopathic gallstone disease in pediatric patients of a North American tertiary care center

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Abstract

AIM: To assess spectrum and etiology of gallstones and biliary sludge in the pediatric population of a North American tertiary care centre.

METHODS: Retrospective review of abdominal ultrasounds recorded at Saint Justine Hospital over a period of 24 mo (8/2003 to 8/2005) in patients < 19 years of age. Patients < 2 years of age were analyzed separately.

RESULTS: The presence of gallstones was noted in 127 patients. In 107 it was a new diagnosis, in 48/105 (45.7%) patients > 2 years of age idiopathic gallstone disease was found. These 48 patients represent 2.1% of the population who required ultrasound for abdominal pain. Complicated gallstone disease occurred in 28/48 with idiopathic disease, mainly adolescent girls. Patients with hemolytic disorders, cystic fibrosis, oncologic diseases or kidney transplantation and gallstones were asymptomatic and stones were detected during routine abdominal ultrasound. Twenty two patients < 2 years of age not consulting for abdominal pain had gallstone disease of diverse etiology. Biliary sludge was seen in 84 patients, 78.5% on total parenteral nutrition. In 4 patients, sludge progressed to gallstones.

CONCLUSION: Idiopathic gallstone disease and its rate of complication are more frequent in our cohort than expected from previous studies. Adolescent girls with abdominal pain and idiopathic gallstones require special attention for complicated disease course.

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Key words: Cholecystectomy; Hemolytic disease; Cystic fibrosis; Pancreatitis; Choledocholithiasis

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INTRODUCTION

The rising prevalence of gallstone disease in the adult populations of industrialized countries is a cause of concern. Over 10% of European and American population carry gallstones^[1]. European studies in pediatric patients estimate an overall prevalence of 0.13% to 1.9% for gallstone disease in children up to 19 years of age^[2,3]. However, no details on the etiology or the occurrence of symptoms and complications, as have been published for adult patients with gallstone disease^[4], are known from the pediatric cohorts. Furthermore, only scarce prevalence data for the North American pediatric population is available. In the light of this situation we decided to assess the types, predisposing diseases, modes of presentation and therapies of gallstone disease and biliary sludge in a North American pediatric tertiary care centre.

MATERIALS AND METHODS

We reviewed the records of all complete abdominal ultrasounds, where the result of the inspection of the upper abdominal site was mentioned in the written report, and which were carried out for abdominal pain, for clinically indicated follow-up of diverse abdominal pathologies, malformation screening or urinary tract infection, in patients < 19 years of age, at Saint Justine Hospital over a period of 24 mo (8/2003 to 8/2005). Repeated ultrasounds in the same patient and for the same pathology were classified as follow-up exams. Charts of patients found to carry gallstones, or biliary sludge were reviewed, and the patients divided in 2 groups: 2 years and > 2 to < 19 years of age. This division appeared to

be logical because patients < 2 years of age were followed for diseases present from the perinatal period onwards. We also reviewed the charts of all patients undergoing cholecystectomy during the study period. Complicated biliary pain was considered an indication for laparoscopic cholecystectomy, which was usually carried out after normalization of liver and pancreatic serum parameters. A first episode of uncomplicated biliary pain was an indication for elective cholecystectomy only after exclusion of other etiologies for right upper quadrant pain with similar duration and nausea. Underlying diseases, weight and height, family history of cholelithiasis, serum lipids repeatedly recorded as elevated and done in the fasting state, alanine aminotransferase, aspartate aminotransferase, gamma-glutamyl transferase, total and direct bilirubin, amylase, lipase as well as the pathology results of cholecystectomy specimens and outcome as far as available were analyzed.

Definitions

Patients with gallstones and no associated disease were defined as having idiopathic gallstone disease. Cholelithiasis was defined as an echogenic focus producing acoustic shadowing in the gallbladder or in the region of the gallbladder. Biliary sludge was defined as a non-shadowing, echogenic, intraluminal sediment. Biliary pain was defined as paroxysmal abdominal pain with right upper quadrant tenderness increasing over 15 min and lasting maximal 6 h^[5]. Pancreatitis was defined as elevation of serum amylase > 3 times the normal value and abdominal pain. Cholangitis included cholestasis, fever, and upper abdominal tenderness^[6]. Elevation of direct and total bilirubin beyond the upper limit of normal for age was considered a sign of biliary obstruction, with or without additional elevation of gamma glutamyl transpeptidase, alanine aminotransferase and aspartate aminotransferase. Overweight was defined as body mass index (BMI) > 95th, tendency for overweight as BMI > 85th percentile^[7].

Statistical analysis

All testing was based on determining statistical significance at a 2-sided α level of 0.05. The *Kolmogorov-Smirnov* test was used to flag normal distribution. The 2-sample *t*-test served to compare frequency distributions between subgroups, with the *Mann-Whitney U* test for comparison of groups without normal distribution. The study was approved by the Research Ethics Committee of Saint Justine Hospital.

RESULTS

A total of 13259 complete abdominal ultrasounds in 9203 patients were included and reviewed. Of these, 2218 were done for abdominal pain with or without further clinical specification. Routine follow-up ultrasounds were carried out in patients with hemolytic disorders, cystic fibrosis, chronic liver and intestinal diseases, oncologic diseases or transplanted patients. Age distribution of patients with gallstones detected and the indication of the ultrasound are shown in Table 1. While reviewing all these ultrasounds we also found biliary sludge in 84 patients (0.91%). Table 2

Table 1 Indications for abdominal ultrasounds in < 2 or 2-19 years old patients with gallstone disease

Ultrasound	For abdominal pain (n = 55)		For follow-up (n = 72)		Total (n = 127)	
New diagnosis	No	Yes	No	Yes	No	Yes
> 2 yr	4	51	16	34	20	85
< 2 yr	0	0	0	22	0	22

shows the associated diseases and age distribution of patients with gallstone disease and sludge. A minority of patients with gallstones or sludge required radiological examination for indications other than biliary tract disorders and data on radiopacity of the gallstones are not available.

Associated disorders in patients older than 2 years of age

Idiopathic gallstones: Idiopathic gallstone disease was found in 48/105 (45.7%) patients > 2 years of age (33 girls with a median age of 15 years, and 15 boys with a median age of 12 years, 35.4% of non French or English origins: immigrants from the South American continent (8), Eastern and Southern Europe, Russia, Arabic or Far East countries), 2.1% of all patients requiring ultrasound for abdominal pain. Biliary pain was the reason for an abdominal ultrasound in 19/48 patients, and unspecified abdominal pain in 29/48. The male to female ratio was 1:2. Body mass index > 85th percentile was noted in 14/48 (29.2%, all girls); family history of gallstone disease and cholecystectomy was positive in 25, negative in 3, and not recorded in 20/48 patients. Gallstones evolved with complications such as elevated serum bilirubin and serum amino-transferases without visible choledocholithiasis (13), with pancreatitis (13) and/or choledocholithiasis (8) in 28/48 patients. All had cholecystectomy, 27 within 4 wk after the end of the acute episode, and one pregnant patient after delivery (Table 3). Endoscopic retrograde cholangio-pancreatography and papillotomy for symptomatic and obstructive common bile duct stones were carried out before cholecystectomy in 4/28 patients and intra-operative cholangiography in one. The pathological analysis of cholecystectomy specimens was limited to the description of stone colour and gallbladder wall histology. Stones were described as yellow, pale or yellowish green in 24/28 patients, and as black or predominantly black in 4/28 (4 boys). Gallbladder histology was normal in 3, with signs of chronic cholecystitis in 25/28, and of cholesterosis in 8 of these 25. In patients with cholesterosis (2 boys, 6 girls, 12-18 years old at cholecystectomy), serum cholesterol was normal in 3 and not tested in 5. BMI was > 85th percentile in 2/28 patients.

Of the 20/48 patients with abdominal pain and no complications or cholecystectomy (Table 3), 9/20 were lost for follow-up, 11 had regular follow-up for other conditions (asthma in 6, epilepsy in 2, orthopedic problems in 3), and no recurrence of abdominal pain was recorded. Only 2/11 patients were on ursodeoxycholic acid. Positive family history of gallstone disease and cholecystectomy was found in 3, negative in 2, not recorded in 15. BMI was > the 90th and > 95th percentile in 2 patients each.

Table 2 Numbers of patients with gallstones or sludge and associated diseases

Age (median, range)	Hemolytic	CF	Oncologic/transplantation	Infectious	Surgery + TPN	Idiopathic	Total	Male patients (% total patient number)
Gallstones								
< 2 yr (2 mo, 0.5-17)				12	10		22	11 (50)
> 2 - < 19 yr (13 yr, 3-18)	19	10	10	8	10	48	105	37 (35)
Sludge								
< 2 yr (2 mo, 0.2-16)	2	2	31	9	12		28	18 (64.3)
> 2 - < 19 yr (13 yr, 2.3-17)	12	4	121	10 + 1 ¹	14	3	56	23 (41.1)
Total	33	16	25	40	46	51	211	89 (42.2)

CF: Cystic fibrosis; TPN: Total parenteral nutrition; ¹Plus TPN.

Table 3 Age, gender and associated diseases in 2-19 years old patients with and without cholecystectomy

	Cholecystectomy, <i>n</i> = 37 (median age, yr)			No cholecystectomy, <i>n</i> = 68 (median age, yr)		
Associated disorders and gender (female/male)	Female	Male	%	Female	Male	%
Idiopathic	23 (14.5 ¹)	5 (13 ¹)	58	10 (16 ¹)	10 (10.5 ¹)	42
Hemolytic	4 (13 ¹)	5 (9.5 ¹)	47	4 (16 ¹)	6 (11 ¹)	53
Cystic fibrosis	0	0	0	7 (15.5 ¹)	3 (15 ¹)	100
Oncologic	0	0	0	8 (9 ¹)	2 (9 ¹)	100
Infectious/TPN/surgery	0	0	0	13 (12 ¹)	5 (11 ¹)	100

¹Age differences between male and female patients not significant.

Hemolytic disorders: Hemolytic disorder was present in 19 (18%) patients > 2 years of age with gallstones (sickle cell disease in 11, hereditary spherocytosis in 6, hemolytic uremic syndrome and autoimmune hemolytic anemia in 1 each), and 3/19 had abdominal pain requiring ultrasound (Table 1 and Table 3). No genetic testing for Gilbert disease had been performed in these patients^[8]. Preventive cholecystectomy was performed in 9/19 and for gallstone disease with choledocholithiasis in 1 patient only.

Cystic fibrosis (CF): None of the 10 patients with CF and gallstone disease had complicated disease, and there was no cholecystectomy (Table 3).

Oncologic diseases or post-transplantation status: Ten patients with oncologic diseases or post-transplantation status (Table 3) were found to carry gallstones and no complication occurred and no cholecystectomy was required.

Diverse diseases: Multifactorial and chronic disorders were present in 18 patients > 2 years of age and the gallstones were associated with total parenteral nutrition (TPN) or intestinal disorders (Crohn's disease, lymphangiectasia) in 7, hepatic disease in 4, and recurrent, severe infections in 7 patients.

Associated disorders in patients younger than 2 years of age

In total, 22 (17.3%) patients < 2 years of age had gallstones. The underlying diseases were infections (12), diseases requiring TPN (7), or congenital hepatic disease [progressive familial intrahepatic cholestasis (1), malformation of the common bile duct (2)] (Table 1). All patients had clinical

follow-up visits, but only 2 required follow-up ultrasound for pain. Eventual stone disappearance was not documented.

Biliary sludge: Biliary sludge was found in 84 patients, and 66/84 (78.5%) had TPN at the time of, or until 2 wk before the diagnosis of biliary sludge. 28/84 patients were < 2 years of age (21 TPN, 5 cardiopathies, 2 CF). 56/84 patients were > 2 years of age (8 hemolytic disorders, 31 TPN, 13 severe infections, 4 CF). Biliary sludge evolved with complications, such as bile duct dilatation (2), pancreatitis (12) or thickened gallbladder wall (7) in 21/84 patients (25%, 6 < 2 and 15 > 2 years of age). Cholecystectomy was carried out in 2 patients, in 1 with recurrent pancreatitis, and 1 with sickle cell disease and suspicion of acalculous cholecystitis. Progression from biliary sludge to gallstone disease was documented in 4/84 patients (4.7%), 1 each with sickle cell disease, CF, hemolytic uremic syndrome and sepsis.

DISCUSSION

Our study revealed 127 patients with gallstone disease: 105 were older than 2 years of age, and about 50% of these had idiopathic gallstone disease. Idiopathic gallstone disease was therefore found in 2.1% of all patients having an abdominal ultrasound for abdominal pain. Half of the patients with idiopathic gallstone disease presented with complications such as cholestasis, choledocholithiasis and/or pancreatitis. Most patients with idiopathic gallstone disease, and most of those with complications were adolescent girls. Furthermore, over 50% of patients > 2 years of age were symptomatic at the time of stone detection, and the majority had idiopathic gallstone disease. In contrast, patients with gallstones of defined etiologies often were not symptomatic.

Comparability of our results with those of recently published studies was limited by discrepancies in the study duration and patient selection. The work with the most comparable design, an European study recently published by Wesdorp *et al*^[2], had to go through 11 years' data (1988-1999) to review a similar number of abdominal ultrasounds as we had the opportunity to accumulate in a 2 years interval (2003-2005). They found idiopathic disease in 23% of their gallstone carriers. No comparison was possible with the Italian survey carried out by Palasciano *et al*^[3], because this study included only healthy children. We therefore reverted to older data^[9-11], where temporary conditions such as sepsis, TPN, trauma, antibiotics or immobilization were found in 22% of children with gallstone disease, and other specific conditions, such as short bowel disease, inflammatory bowel disease, and cystic fibrosis in 38%. In those cases, up to another third of children suffered from hemolytic diseases^[1]. The gallstone rate in our patients with hemolytic disorders was similar to that of the North American surveys^[9,10], whereas a much lower rate was found in Europe^[2]. Differing prevalence of Gilbert syndrome may explain this discrepancy^[8,9]. The gallstone rate in patients with cystic fibrosis or Crohn's disease was lower in our cohort than in previous pediatric studies^[9], but similar to that of the adult North American patient population^[12,13], and the European studies. The lower prevalence of these presumed pigment stones and the lack of complications may reflect generally improved treatments for both diseases.

The most noticeable finding, however, was the larger proportion of idiopathic gallstone disease in patients > 2 years of age when compared to the results of the Wesdorp's study (45.7% *vs* 23%), and also in comparison to older North American pediatric surveys^[9-11]. Furthermore, more than half of the patients with idiopathic disease presented with complications, such as pancreatitis or elevated liver enzymes and/or elevated serum bilirubin, and they all required cholecystectomy. The majority were adolescent girls, and nearly all had yellowish, presumably mixed cholesterol stones. In contrast, the majority of the few male patients in the idiopathic gallstone group with cholecystectomy had black or dark colour stones, and the patients without cholecystectomy classed as idiopathic gallstone disease had an equal gender distribution. We therefore conclude that adolescent female patients with complicated gallstone disease and yellowish gallstones are a patient group with adult type of idiopathic gallstone disease^[13,14], as opposed to the remaining patients who may have diverse etiologies, as previously described^[15,16].

Patients < 2 years of age (17.3%) were examined as a separate group, because in most cases their conditions had been requiring medical care since birth. Their lithiasis evolved asymptotically, with no cholecystectomy or long-term sonographic follow-up required, and their stones were assumed to be of infectious origin. No gender difference was found in this age group and the rates found were comparable with those published by others^[1,17].

Biliary sludge occurred without gender difference, was equally distributed in all age groups and was associated with a lack of enteral feeding due to surgery, intestinal

disease requiring parenteral nutrition, hemolysis or systemic infections. The rate found in our study was similar to that of adult patients^[18-20]. Complications such as pancreatitis and dilatation of the common bile duct occurred in 25%, which is more than expected^[21,22]. The high prevalence of complications can be attributed to the high proportion of chronically- and severely-ill patients hospitalized at Saint Justine Hospital. However, cholecystectomy was only exceptionally indicated. The rate of progression of sludge to gallstone disease was comparable with that of other studies^[20].

In summary, a higher than expected rate of idiopathic gallstone disease was detected in our patient cohort. Complications such as pancreatitis, elevated liver enzymes and/or elevated serum bilirubin or choledocholithiasis requiring cholecystectomy occurred more frequently than expected. The patients afflicted were mainly adolescent girls, a third of them of non-French Canadian origin. Pediatricians should be aware of the increasingly frequent adult type gallstone disease occurring in adolescent girls presenting with abdominal pain. Further research is necessary to predict children at risk for complicated idiopathic gallstone disease and prevent complications before cholecystectomy.

COMMENTS

Background

The prevalence of gallstones in adults of industrialized countries approximates 10% and shows a tendency to rise. Prevalence data in pediatric patients are scarce.

Research frontiers

Current doctrine teaches that adult gallstone disease is predominantly idiopathic and most stones are composed of cholesterol, whereas the same disease in pediatric patients is due to specific causes like infectious diseases, and intestinal, hepatic or haemolytic disorders, and stones are predominantly composed of bilirubin polymers and calcium salts.

Innovations and breakthroughs

We found 2.1% patients ($n = 48$) with idiopathic gallstone disease as new diagnosis, mainly adolescent girls, 35% of other than French Canadian origin among patients requiring abdominal ultrasound for abdominal pain. Of these 48 patients, 58% presented with complications such as pancreatitis, cholestasis or choledocholithiasis. Idiopathic gallstone disease and complicated disease presentation were much more frequent than expected from previous studies.

Applications

Pediatricians should become more aware of a disease until now attributed to adult patients only. In the light of the recent detection of a human lith gene ABCG5/G8 and its association with the risk for cholesterol gallstone formation, the value of genetic evaluations of such families in order to prevent complicated disease presentation should be discussed.

Terminology

Gallstones of unknown etiology are referred to as idiopathic gallstone disease, in contrast to gallstones of known etiology, such as for instance hemolytic disorders.

Peer review

This is a well written and informative paper. The study estimated the prevalence of gallstones in the pediatric population (9203 patients < 19 years of age) of Canadian tertiary care centre by reviewing abdominal ultrasounds recorded over two years. Gallstones were found in 127 patients, 50% of these were considered idiopathic which was more than expected from previous studies.

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High circulating D-dimers are associated with ascites and hepatocellular carcinoma in liver cirrhosis

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Abstract

AIM: To measure plasma D-dimer levels in cirrhotic patients with and without ascites, assessing the effect of ascites resolution in D-dimer concentration.

METHODS: Seventy consecutive cirrhotic patients (M = 44, F = 26, mean age 65 years, SD \pm 13), observed from October 2005 to March 2006 were enrolled. Circulating D-dimer levels were measured using a latex-enhanced, immunoturbidimetric test. In patients with ascites (n = 42) the test was repeated after ascites resolution.

RESULTS: Ascites was present in 42 patients (group A) and absent in 28 (group B). Group A patients had more advanced liver disease. Hepatocellular carcinoma (HCC) was diagnosed in 14 patients and was more frequent in group B. Above normal range D-dimers were found in 45/70 patients. High D-dimers were more frequent in group A than in group B (P = 0.001). High D-dimers were associated with presence of HCC (P = 0.048) only in group B. After ascites resolution, obtained in all patients, mean D-dimer values decreased in those 34 patients with high basal levels (P = 0.007), returning to normal in 17.

CONCLUSION: In patients with liver cirrhosis, ascites and HCC are the main factors associated with increased fibrinolytic activity.

INTRODUCTION

Hemostasis is a dynamic process resulting from the balance between procoagulant and anticoagulant factors. The liver is the site of production of most proteins which favour and inhibit the process of coagulation and fibrinolysis. Patients with liver cirrhosis may develop a serious coagulopathy whose origin is commonly ascribed to a defective hepatic synthesis of clotting factors often in association with thrombocytopenia secondary to portal hypertension. In addition, patients with advanced liver disease have a hyperfibrinolytic state which contributes to the bleeding tendency causing a premature removal of the hemostatic plug. Plasma levels of fragment D-dimer represent an accurate marker of fibrinolytic activity. The finding of high D-dimer plasma concentration in patients with liver cirrhosis, decompensated by ascites, led Agarwal *et al*^[1] to suggest a major role of ascites in the pathogenesis of hyperfibrinolytic state associated with liver failure. The aims of this study were: (1) to evaluate the relationship between the presence of ascites and hyperfibrinolytic state in liver cirrhosis measuring the circulating levels of D-dimer in patients with and without ascites and (2) to assess the effect of ascites resolution in the plasma concentration of D-dimers.

MATERIALS AND METHODS

Subjects

The study was designed to measure plasma D-dimer levels in all consecutive patients with cirrhosis of the liver referring to the Unit of Clinica Medica, Messina University,

Table 1 Clinical and demographic features of the patients with liver cirrhosis

	Ascites <i>n</i> = 42	No ascites <i>n</i> = 28	<i>P</i> value
Gender M/F	24/18	20/8	0.735
Mean age \pm SD (yr)	67 \pm 14	64 \pm 14	0.068
Child-Pugh class (%)			0.004
A	8 (19.0)	18 (64.3)	
B	18 (42.9)	8 (28.6)	
C	16 (38.0)	2 (7.1)	
Patients with HCC (%)	2 (4.8)	12 (42.8)	0.000

Italy, during a 6 mo period (October 2005-March 2006), with the exclusion of patients with deep venous or portal vein thrombosis. We included 70 patients and excluded 1 patient with portal vein thrombosis. Informed consents were obtained from all patients. The diagnosis of cirrhosis was either histological or made by clinical and/or imaging signs of portal hypertension. The etiology of liver disease was HCV infection in 31 patients, HBV infection in 4, past alcohol abuse in 15, autoimmune in 2, HBV + HCV coinfection in 1, primary biliary cirrhosis in 1, HCV + alcohol abuse in 2 and cryptogenic in 14. Hepatocellular carcinoma (HCC) was diagnosed in 14 patients. All patients underwent color-Doppler ultrasonographic study of the abdomen and the legs. Severity of liver disease was classified by Child-Pugh score. Patients were divided into 2 groups according to the presence of ascites. Patients with ascites were treated with fluid and salt restriction, diuretics and/or paracentesis. The resolution of ascites was confirmed by ultrasonography. D-dimer levels were measured also after disappearance of abdominal fluid. The demographic and clinical features of the patients studied are shown in Table 1.

Blood collection and testing

Peripheral blood was collected into tubes containing sodium citrate solution. After centrifugation (10 min, 1500 \times g), the supernatant plasma was removed. Plasma D-dimer was measured by a latex-enhanced, immunoturbidimetric test using a commercially available kit (D-dimer PLUS, Dade Behring, Marburg, Germany). The D-dimer concentration was expressed in μ g/L with a normal range of 125-350 μ g/L.

Statistical analysis

Differences between groups were evaluated by non parametric permutation Test (NPC test) for numerical variables and by Anderson Darling Test for categorical variables^[2]. Association between D-dimer values and gender of patients, Child-Pugh class and HCC presence was analyzed by Log-Likelihood ratio test^[3]. Correlation between D-dimer values and age of patients was evaluated using biserial correlation coefficient^[4]. Influence of the variables, gender, age, Child-Pugh class and HCC presence on D-dimer values was estimated by a logistic regression model^[5]. Softwares SPSS, Windows 11.0 (2001) for Binary Logistic Regression, Microsoft Excel (2002) for *G* test, Methodologica S.R.L. (2001) for nonparametric analysis NPC test and Anderson Darling test were used.

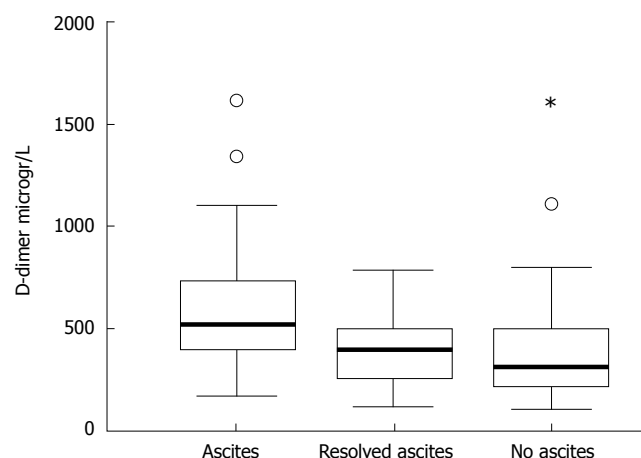


Figure 1 Distribution of plasma D-dimer in patients with liver cirrhosis. *Indicates outlier between 1.5- IQR (Interquartile Range) and 3-IQR below Q1 (first quartile); *Indicates outlier more than 3-IQR below Q1.

RESULTS

Ascites was present in 42 patients (group A) and absent in 28 (group B). No significant differences in age and gender were found between the two groups. Patients of group A had more advanced liver disease. HCC was more frequent in patients of group B (Table 1). Plasma D-dimer levels above the normal range were found in 45/70 patients (64.3%). D-dimer above normal values were more frequent ($P = 0.001$) in group A (34/42) than in group B (11/28). D-dimer mean values were higher ($P = 0.001$) in group A (649 ± 420 g/L) than in group B (359 ± 219 g/L, Figure 1). In all patients of group A resolution of ascites was obtained either by fluid and salt restriction and conventional diuretic treatment (in 36) or by paracentesis (in 6). After disappearance of ascitic fluid, confirmed by ultrasonography, mean D-dimer values decreased in all 34 patients with high basal levels ($P = 0.007$), returning to normal range in 17. In these patients, D-dimer values after resolution of ascites (438 ± 279 g/L) were not significantly different from those found in patients without ascites, group B, (Figure 1).

In the whole population of patients or the separate groups A and B, high D-dimer levels were not associated with gender neither with Child-Pugh class (Anderson-Darling test) and did not correlate with anagraphic age. Only in patients without ascites, group B, high D-dimer levels were associated with presence of HCC ($P = 0.048$). When we inserted the variables age, gender, Child-Pugh class and presence of HCC in a logistic regression model (where the response variable was represented by normal or high D-dimer class), we found that high levels of D-dimer were significantly dependent on the presence of HCC ($P = 0.030$) in patients without ascites (group B) and negatively dependent on age ($P = 0.019$) in patients with ascites (Group A).

DISCUSSION

Patients with liver cirrhosis have a bleeding tendency that is often not evident from routine clotting studies. In such patients, the incidence of hyperfibrinolysis varies from 19% to 95%^[6-8] and may contribute to serious bleeding

complications. The pathogenesis of hyperfibrinolysis in liver cirrhosis is not yet clearly known. Having found high D-dimer levels in the blood and in the ascitic fluid, Agarwal *et al* suggested ascites among the possible causes of increased fibrinolysis in patients with liver cirrhosis^[1].

The aim of our study was (1) to evaluate the relationship between the presence of ascites and hyperfibrinolytic state in cirrhotic patients measuring the circulating levels of D-dimer in those with and without ascites and (2) to assess the effect of ascites resolution in the concentration of D-dimers. High D-dimer levels in 64% of patients with liver cirrhosis were observed. This finding is in agreement with that of Agarwal *et al* who reported increased plasma D-dimers in 63% of patients with liver cirrhosis^[1]. This percentage is higher than that reported in another study where abnormal D-dimers were found only in 17% of patients with chronic liver disease. However, 11 out of 86 patients included were not cirrhotic and the number of ascitic patients was not specified^[9]. When we divided our patients into two groups, according to the presence of ascites, we found high D-dimers in 81% of patients with ascites and in 39% of patients without. This finding is also consistent with that of Agarwal *et al* who showed increased plasma D-dimer values in 93% and 33% of patients with and without ascites, respectively^[1]. Furthermore, our patients with ascites had mean D-dimer values significantly higher than those without. After resolution of ascites, circulating D-dimers decreased significantly in all patients, returning to normal in half of them. Also, mean D-dimer levels in cirrhotic patients with resolved ascites were not significantly different from those in patients who entered the study without ascites. Therefore, our data confirm the association between circulating high D-dimer levels and the presence of ascites found in cirrhotic patients.

Another interesting finding of our study is the close association between presence of HCC and high D-dimer values in patients without ascites. HCC is often associated with thrombotic invasion of portal or hepatic vein. Although we excluded from the study all patients with clinical and imaging features of thrombosis, we cannot rule out the presence of microvascular invasion in patients with HCC. In fact, Kim *et al*^[10] also found increased circulating D-dimers in patients with HCC, even in absence of tumor thrombosis in a major branch of the portal or the hepatic vein.

The underlying mechanism for high D-dimer plasmatic levels in cirrhotic patients with ascites remains to be clarified. Some authors suggest the exchange of some coagulation and fibrinolytic proteins between plasma and ascitic fluid^[11,12]. Violi *et al* propose that hyperfibrinolysis in cirrhotic patients might represent a state of low grade disseminated intravascular coagulation secondary to the passage of gut absorbed bacterial material into the systemic circulation^[12]. On the basis of this finding, Piscaglia *et al* argue that the association between high plasma D-dimers and ascites might be due only to more advanced liver disease with portal hypertension favouring bacterial translocation. In our study, however, we were not able to demonstrate any correlation between circulating D-dimers and severity of disease^[13].

Although some of our patients with high D-dimers had no ascites, it must be underlined that 64% of them had HCC. In conclusion, our study shows that high D-dimers are associated either with presence of ascites or with HCC. In patients with liver cirrhosis, high D-dimer levels in absence of ascites require more careful monitoring for HCC. Depletion of ascitic fluid might prevent bleeding complications, especially if invasive procedures become necessary.

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COMMENTS

Background

Patients with advanced liver disease have a hyperfibrinolytic state which contributes to the bleeding tendency causing a premature removal of the hemostatic plug. Plasma levels of fragment D-dimer represent an accurate marker of fibrinolytic activity.

Research frontiers

The finding of high D-dimer plasma concentration in patients with liver cirrhosis, decompensated by ascites, led Agarwal *et al* to suggest a major role of ascites in the pathogenesis of hyperfibrinolytic state associated with liver failure.

Innovations and breakthroughs

High D-dimer plasma levels in patients with liver cirrhosis are associated with presence of ascites and decline after its resolution. In absence of ascites, high D-dimer values are associated with presence of hepatocellular carcinoma (HCC).

Applications

In patients with liver cirrhosis depletion of ascitic fluid might prevent bleeding complications, especially if invasive procedures become necessary. In absence of ascites, high D-dimer levels rise suspect of HCC and require more careful monitoring.

Terminology

Fragment D-dimer is a degradation product from a specific region of cross-linked fibrin. High plasma D-dimer levels represent a sensitive but not specific marker of fibrinolysis and thrombosis.

Peer review

This was a modest but interesting paper with some potential clinic significance.

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Impact of obesity on the surgical outcome following repeat hepatic resection in Japanese patients with recurrent hepatocellular carcinoma

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Abstract

AIM: To evaluate the impact of obesity on the postoperative outcome after hepatic resection in patients with hepatocellular carcinoma (HCC).

METHODS: Data from 328 consecutive patients with primary HCC and 60 patients with recurrent HCC were studied. We compared the surgical outcomes between the non-obese group (body mass index: BMI < 25 kg/m²) and the obese group (BMI ≥ 25 kg/m²).

RESULTS: Following curative hepatectomy in patients with primary HCC, the incidence of postoperative complications and the long-term prognosis in the non-obese group (*n* = 240) were comparable to those in the obese group (*n* = 88). Among patients with recurrent HCC, the incidence of postoperative complications after repeat hepatectomy was not significantly different between the non-obese group (*n* = 44) and the obese group (*n* = 16). However, patients in the obese group showed a significantly poorer long-term prognosis than those in the non-obese group (*P* < 0.05, five-year

survival rate; 51.9% and 92.0%, respectively).

CONCLUSION: Obesity alone may not have an adverse effect on the surgical outcomes of patients with primary HCC. However, greater caution seems to be required when planning a repeat hepatectomy for obese patients with recurrent HCC.

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Key words: Body mass index; Hepatocellular carcinoma; Hepatectomy; Prognosis

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INTRODUCTION

Obesity, the incidence of which has recently been growing at an epidemic rate in Japan^[1], as well as in the other Western countries, has been implicated as a risk factor for postoperative complications in general surgery^[2-4]. Indeed, several studies have shown that obesity increases the risk of wound complications in various types of surgical procedure^[5]. However, the effects of obesity on surgical outcomes remain controversial^[6-8].

The World Health Organization (WHO)^[9] defines obesity as a body mass index (BMI) above 30 kg/m², but in Japan, the prevalence of the population with such a BMI is no more than 2%-3%, in contrast to the 20%-30% prevalence in Western countries^[10]. In Japan, the definition

of obesity is proposed to be a BMI above 25 kg/m², since the incidence of obesity-related disorders increases with a BMI \geq 25 kg/m²[1,11]. On the other hand, recent epidemiological studies have demonstrated that obesity is a risk factor for hepatocellular carcinoma (HCC)[12-14]. To our knowledge, however, there are no data regarding the postoperative outcomes in obese patients with HCC. Therefore, in the present study, we specifically evaluated the influence of obesity (BMI \geq 25 kg/m²) on the early and late surgical results following curative hepatic resection in Japanese patients with HCC.

MATERIALS AND METHODS

From January 1995 to August 2006, 328 consecutive patients with HCC received a curative primary hepatic resection at Hiroshima Red Cross Hospital and Atomic Bomb Survivors Hospital. Sixty patients with intrahepatic recurrence, who underwent a curative repeat hepatectomy, were also examined. A curative hepatectomy indicates that no tumor remained in the remaining liver based on the findings of an intraoperative ultrasound examination or a computed tomographic (CT) study performed within 3 mo after surgery. Repeat hepatectomy for recurrent HCC was considered to be the treatment of choice for resectable recurrent tumors. Our selection criteria for repeat hepatectomy were the same as those for primary resection for HCC. The selection of type of hepatectomy was made on the basis of tumor location and liver function[15]. Patients with uncontrollable ascites are considered to be contraindicated for a hepatic resection. Therefore, no patients with ascites were included in this study. Height and weight were measured preoperatively, and the BMI (kg/m²) was calculated as weight (kg) divided by squared height (m). Although the WHO[9] defines obesity as a BMI above 30 kg/m², only 7 (2.1%) of the 328 patients with primary HCC and 1 (1.7%) of the 60 patients with recurrent HCC met this definition. Therefore, in the present study, we defined obesity as a BMI \geq 25 kg/m².

The records of 328 patients with primary HCC were reviewed retrospectively. These patients were classified into two groups based on BMI: an obese (BMI \geq 25 kg/m²) group and a non-obese (BMI < 25 kg/m²) group. Similarly, the 60 patients with recurrent HCC were classified into two groups based on BMI. We compared clinicopathological parameters, such as liver function data, operative data, postoperative complications and tumor factors, between the obese group and the non-obese group. Postoperative complications were defined as any event that required specific medical or surgical treatment. We also compared the survival rates and disease-free survival rates between the two groups. All patients were closely followed after operation. Monthly measurements of alpha-fetoprotein and protein induced by vitamin K absence-II (PIVKA-II) were performed. Every 3 to 4 mo, ultrasonographic examination and dynamic CT were performed by radiologists. An angiographic examination was performed after admission when recurrence was strongly suspected.

Statistical analysis

To compare the clinicopathological variables between the

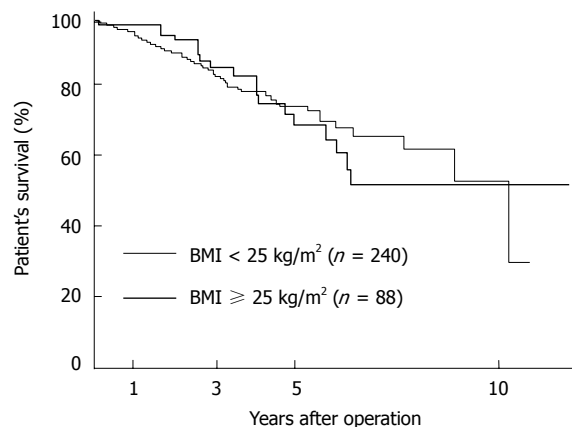


Figure 1 Comparison of the survival curves after a primary hepatectomy in the nonobese group (BMI < 25 kg/m², *n* = 240) versus the obese group (BMI \geq 25 kg/m², *n* = 88). The patient's survival rate in the nonobese group did not differ significantly from that in the obese group.

two patient groups, either the unpaired Student's *t* test or the chi-square test was used. Survival was estimated by the Kaplan and Meier method and the differences in survival between the groups were then compared using the log-rank test. Only a few significant variables were analyzed in the multivariate analysis using Cox's proportional hazard model. A *P* value < 0.05 was considered to be significant.

RESULTS

Impact of obesity (BMI \geq 25) on hepatectomy for primary HCC

Table 1 summarizes the clinicopathological variables of the patients in the non-obese (BMI < 25 kg/m², *n* = 240: 73%) and obese groups (BMI \geq 25 kg/m², *n* = 88: 27%). The percentage of female patients in the obese group was significantly higher than that in the non-obese group (*P* < 0.05). However, the percentage of patients with diabetes mellitus was not significantly different between the two groups. No differences were noted between the groups in terms of liver function data, operative data (such as operation time, blood loss and postoperative complications) or tumor factors. The incidence of postoperative infectious complications in the obese patients (7/88, 8.0%) tended to be higher (*P* = 0.092) than that in the non-obese patients (8/240, 3.3%). However, the incidence of other complications, such as intractable ascites and bile leakage, did not differ significantly between the two groups. In total, the incidence of postoperative complications in the obese group was comparable to that in the non-obese group (Table 1).

The survival of patients in the obese group was almost identical to that in the non-obese group (Figure 1). Moreover, no significant difference was found in the disease-free survival rates between the two groups (Figure 2).

Impact of obesity (BMI \geq 25) on repeat hepatectomy for recurrent HCC

Table 2 compares the clinicopathological features between the non-obese (BMI < 25 kg/m², *n* = 44: 73%) and obese groups (BMI \geq 25 kg/m², *n* = 16: 27%). Similar

Table 1 The clinicopathological data of nonobese (BMI < 25 kg/m²) and obese (BMI ≥ 25 kg/m²) patients with hepatocellular carcinoma who underwent a primary curative hepatectomy

Variables	BMI < 25 kg/m ² (n = 240)	BMI ≥ 25 kg/m ² (n = 88)	P value
Age	65.6 ± 9.9	66.0 ± 8.8	0.725
Gender (male:female)	172:68	50:38	< 0.05
Body mass index (kg/m ²)	21.6 ± 2.2	27.1 ± 2.0	< 0.01
Diabetes mellitus (%)	23.1	25.3	0.684
Positive HCV antibody (%)	72.1	73.9	0.748
Liver function data			
AST (international units/L)	54.8 ± 34.7	48.7 ± 24.0	0.132
Albumin (g/dL)	3.8 ± 0.4	3.9 ± 0.5	0.307
Prothrombin time (%)	87.2 ± 15.2	86.9 ± 15.4	0.881
Child-Pugh class (A:B)	222:18	83:5	0.210
Operative data			
Procedures (Hr0/HrS:Hr1/Hr2)	159:81	57:31	0.752
Operation time (min)	208 ± 97	207 ± 80	0.886
Blood loss (g)	534 ± 526	582 ± 596	0.472
Blood transfusion (%)	13.3	18.2	0.730
Postoperative complications (%)	11.7	13.6	0.658
Postoperative hospital death (%)	0.8	1.1	0.801
Tumor factors			
Tumor size (cm)	3.3 ± 2.4	3.0 ± 1.7	0.337
Solitary tumor (%)	71.3	72.7	0.729
Positive fc (%)	69.3	60.7	0.152
Positive vp (%)	51.1	53.6	0.898
Positive im (%)	9.7	9.5	0.970
Well/Moderately/Poorly ¹	39/170/20	13/58/8	0.933
AFP (> 20 ng/mL) (%)	52.7	50.6	0.730

BMI: Body mass index; HCV: Hepatitis C virus; AST: Aspartate transaminase; Hr0: Resection less than subsegmentectomy; HrS: Subsegmentectomy; Hr1: Segmentectomy; Hr2: Bisegmentectomy; fc: Capsular formation; vp: Invasion to portal vein; im, intrahepatic metastasis; AFP: Alpha-fetoprotein.
¹Histologic differentiation of the tumor.

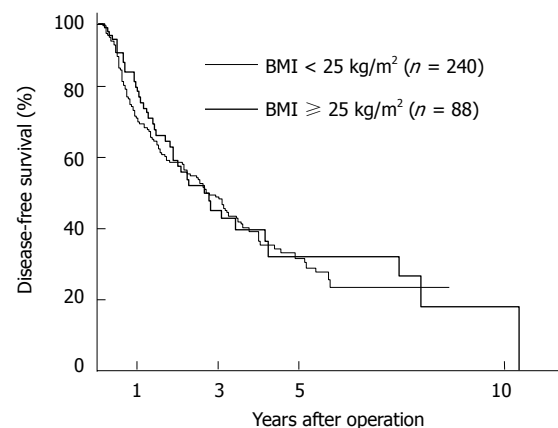
to the patients with primary HCC (Table 1), there was a significantly higher proportion of female patients in the obese group than in the non-obese group ($P < 0.05$). No significant differences were observed in the liver function data and tumor factors. However, the operation time was significantly longer ($P < 0.05$) and the blood loss was significantly greater ($P < 0.01$) in the obese group compared with the non-obese group. Although no statistically significant difference was noted, the patients in the obese group tended to receive blood transfusions more frequently than those in the non-obese group ($P = 0.068$). Four patients (25%) in the obese group had postoperative complications, which included intractable ascites in 2 cases, wound infection in 1 case and angina pectoris in 1 case. The incidence of postoperative complications did not differ significantly between the two groups, and no postoperative hospital death occurred after a repeat hepatectomy.

We also compared the cumulative survival rates, as shown in Figure 3. Patients in the obese group had a significantly poorer prognosis ($P < 0.05$) than those in the non-obese group. The disease-free survival rate in the obese group was also significantly lower than that in the non-obese group ($P < 0.05$; Figure 4). Nineteen of the 44 patients in the non-obese group and 11 of the 16 patients in the obese group had recurrent disease after

Table 2 The clinicopathological data of nonobese (BMI < 25 kg/m²) and obese (BMI ≥ 25 kg/m²) patients with recurrent hepatocellular carcinoma who underwent a repeat hepatectomy

Variables	BMI < 25 kg/m ² (n = 44)	BMI ≥ 25 kg/m ² (n = 16)	P value
Age	67.0 ± 10.3	67.7 ± 7.6	0.809
Gender (male:female)	32:12	7:9	< 0.05
Body mass index (kg/m ²)	21.9 ± 2.0	26.9 ± 1.3	< 0.01
Diabetes mellitus (%)	24.4	20	0.728
Positive HCV antibody (%)	70.5	80	0.463
Liver function data			
AST (international units/L)	39.7 ± 24.7	46.8 ± 31.0	0.367
Albumin (g/dL)	4.0 ± 0.5	3.9 ± 0.4	0.427
Prothrombin time (%)	87.7 ± 13.3	83.6 ± 17.7	0.352
Child-Pugh class (A:B)	43:01:00	14:02	0.136
Operative data			
Procedures (Hr0/HrS:Hr1/Hr2)	40:4	16:0	0.108
Operation time (min)	188 ± 68	243 ± 100	< 0.05
Blood loss (g)	453 ± 336	1044 ± 1120	< 0.01
Blood transfusion (%)	6.8	25	0.068
Postoperative complications (%)	9.1	25	0.128
Postoperative hospital death (%)	0	0	0.999
Tumor factors			
Tumor size (cm)	2.0 ± 0.8	2.4 ± 1.3	0.197
Solitary tumor (%)	72.3	81.3	0.491
Positive fc (%)	42.9	53.3	0.485
Positive vp (%)	42.9	53.3	0.307
Positive im (%)	11.9	0	0.073
Well/Moderately/Poorly ¹	6/29/6	5/9/2	0.384
AFP (> 20 ng/mL) (%)	46.5	60	0.367

BMI: Body mass index; HCV: Hepatitis C virus; AST: Aspartate transaminase; Hr0: Resection less than subsegmentectomy; HrS: Subsegmentectomy; Hr1: Segmentectomy; Hr2: Bisegmentectomy; fc: Capsular formation; vp: Invasion to portal vein; im, intrahepatic metastasis; AFP: Alpha-fetoprotein.
¹Histologic differentiation of the tumor.

**Figure 2** Comparison of the survival curves after primary hepatectomy in the nonobese group (BMI < 25 kg/m², n = 240) versus the obese group (BMI ≥ 25 kg/m², n = 88). No significant difference in the disease-free survival rate between the two groups was observed.

repeat hepatectomy. Regarding the patterns of recurrence, 11 (58%) of the 19 patients in the non-obese group and 2 (18%) of the 11 patients in the obese group had a solitary intrahepatic recurrence. The remaining patients had a widespread recurrence, including multinodular recurrence in the remnant liver and/or extrahepatic recurrence. Therefore, patients in the obese group (9/11, 82%) showed widespread

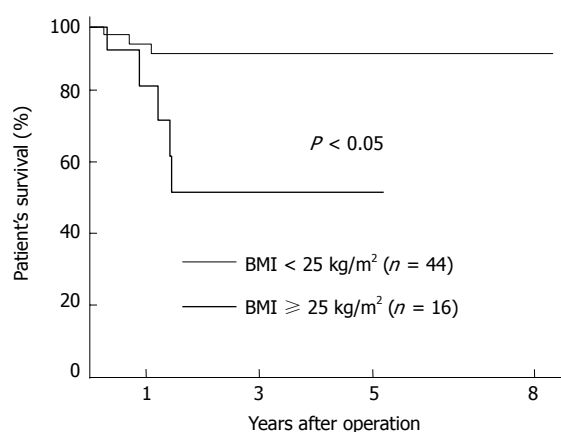


Figure 3 Comparison of the survival curves after repeat hepatectomy in the nonobese group (BMI < 25 kg/m², n = 44) versus the obese group (BMI ≥ 25 kg/m², n = 16). The patient's survival rate in the obese group was significantly lower than that in the nonobese group ($P < 0.05$).

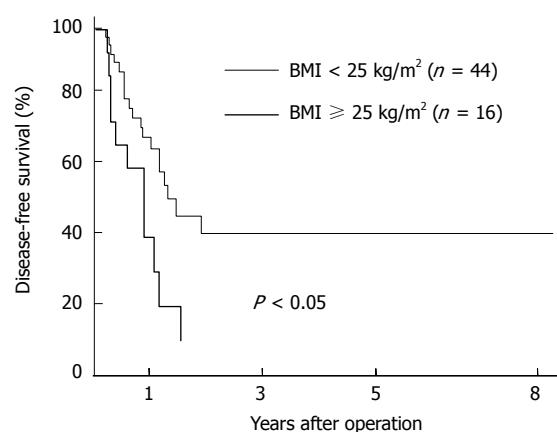


Figure 4 Comparison of the survival curves after a repeat hepatectomy in the nonobese group (BMI < 25 kg/m², n = 44) versus the obese group (BMI ≥ 25 kg/m², n = 16). A significantly shorter disease-free survival was found in the obese group than in the nonobese group ($P < 0.05$).

Table 3 The results of a multivariate analysis using Cox's proportional hazard model in the patients with recurrent hepatocellular carcinoma who underwent a repeat hepatectomy

Variables	Coefficient	SE	Relative risk	P value
Obesity (BMI ≥ 25 kg/m ²)	0.770	0.372	2.160	< 0.05
Tumor size (≥ 2 cm)	0.389	0.438	1.477	0.351
Positive vp	0.466	0.433	1.595	0.254

SE: Standard error; vp: Invasion to portal vein.

recurrences more frequently than those in the non-obese group (8/19, 42%) after a repeat hepatectomy.

In a univariate analysis, 3 variables were identified to be significant prognostic factors: obesity (BMI ≥ 25 kg/m²), tumor size (≥ 2 cm) and microscopic presence of portal vein invasion. Furthermore, a multivariate analysis using Cox's proportional hazard model demonstrated obesity (BMI ≥ 25 kg/m²) to be an independent prognostic indicator in patients with recurrent HCC (Table 3).

DISCUSSION

Obese patients are at an increased risk of numerous medical problems, such as diabetes mellitus, hypertension and immune dysfunction, which can adversely affect the surgical outcomes. However, several studies have investigated this issue and the results are still controversial, possibly due to the use of different definitions and classifications of obesity, and the lack of a uniform way of reporting surgical complications^[16,17]. Dindo *et al*^[6] studied the impact of obesity (BMI ≥ 30 kg/m²) on the outcomes of 6336 patients undergoing general elective surgery. They found that obesity alone was not a risk factor for postoperative complications. Since the association between BMI and health risks to Asian populations is different from that in European populations^[17,18], we defined obesity as BMI ≥ 25 kg/m² in this study. Consistent with the findings of a previous report^[6], there was no significant difference in the incidence of postoperative complications

and postoperative hospital death between the non-obese group (BMI < 25 kg/m²) and the obese group (BMI ≥ 25 kg/m²), following curative hepatectomy in patients with primary HCC. On the other hand, among patients with recurrent HCC, the operation time and blood loss were significantly greater in the obese group than in the non-obese group. Since the operative procedures (extent of hepatic resection) were comparable between the two groups (Table 2), these data reflect the apparent greater technical difficulty of a repeat hepatectomy in obese patients. The wider area of peritoneal adhesion owing to the previous operation and the limited visualization of the surgical field in obese patients might adversely affect the operation time and blood loss. However, the difference in the incidence of postoperative complications after a repeat hepatectomy between the two groups did not reach statistical significance. Therefore, these results indicate that there exists no marked influence of obesity (BMI ≥ 25 kg/m²) on the early surgical outcome after a curative hepatic resection, in both patients with primary HCC and those with recurrent HCC.

Only a few investigators have studied the impact of obesity on long-term outcomes following surgery, and the results are conflicting^[3,19]. Dhar *et al*^[3] suggested that being overweight was an independent predictor of disease recurrence in T2/T3 gastric cancers, whereas Kodera *et al*^[19] reported that obesity did not affect long-term survival after a distal gastrectomy in gastric cancer patients. In the current study, we found no remarkable impact of obesity on the long-term prognosis after a curative hepatectomy in patients with primary HCC. On the other hand, among patients with recurrent HCC, the long-term prognosis following repeat hepatectomy in the obese patients was significantly worse than that in the non-obese patients. Furthermore, obesity (BMI ≥ 25 kg/m²) was an independent poor prognostic indicator in patients with recurrent HCC. The reason why obesity adversely affects the long-term outcome in these patients is not clear. However, several reports have suggested that obesity is significantly associated with a higher rate of death due to many cancers, including liver cancer^[12,13,20,21]. In particular, recent studies have implicated that non-alcoholic

steatohepatitis (NASH), which is characterized by obesity, can progress to liver cirrhosis and HCC^[22-24]. However, pathological examinations in the resected non-cancerous liver specimens revealed only 4 of the 328 patients with primary HCC and one of the 60 patients with recurrent HCC to have NASH in this study. It therefore appears that the poorer prognosis after repeat hepatectomy in obese patients was not attributable to the progression of NASH. The mechanism generally proposed to explain the association between obesity and a worse prognosis include elevated concentrations of insulin-like growth factors^[25], leptin^[26], hormones^[27] and cytokines^[28]. Additional proposed mechanisms include reduced immune functioning and differences in diet and physical activity between obese and non-obese patients^[20,21]. However, these mechanisms fail to explain the poorer prognosis after a repeat hepatectomy in the obese patients, because such a worse prognosis was not observed in obese patients with primary HCC. Among patients with recurrent HCC, those in the obese group showed a significantly greater amount of blood loss and tended to receive blood transfusions more frequently than those in the nonobese group. Many investigators have demonstrated that the increased blood loss and the need for blood transfusions are risk factors for recurrent HCC^[29,30], which is attributed to the induction of immunosuppression. Therefore, the worsened prognosis in obese patients might be associated with the synergistic impacts of the obesity itself and perioperative blood transfusions due to increased blood loss on immune function.

In conclusion, our present findings suggest that the obesity, as defined by a BMI ≥ 25 kg/m², does not have an adverse impact on postoperative outcomes, including postoperative complications and long-term prognosis, after a curative hepatectomy in patients with primary HCC. However, more caution seems to be required when planning a repeat hepatectomy for recurrent HCC in patients who are either overweight or obese.

COMMENTS

Background

Several studies have shown that obesity increases the risk for postoperative complications. However, the influence of obesity on the postoperative outcome after hepatectomy in patients with hepatocellular carcinoma (HCC) has never been evaluated.

Research frontiers

Recent epidemiological studies have demonstrated that obesity is a risk factor for HCC. Therefore, it is important to investigate not only the short-term outcome, but also the long-term outcome after hepatectomy in patients with HCC.

Innovations and breakthroughs

The long-term prognosis after curative resection for HCC remains unsatisfactory, because of a high rate of recurrence. Repeat hepatectomy has been established as the treatment of choice for recurrent HCC. However, our present findings suggest that more caution may be required when planning such a procedure for obese patients with recurrent HCC.

Applications

The authors would like to emphasize that the incidence of obesity-related disorders increases with a BMI ≥ 25 kg/m² in Japan. The prevalence of the population with such a BMI in Japan is almost identical to that of the population with a BMI \geq

30 kg/m² in Western countries. This is the reason why we considered the definition of obesity used in this study to be reasonable. Therefore, it will be valuable to evaluate the impact of obesity on surgical outcomes following repeat hepatectomy in Western countries.

Terminology

The WHO defines obesity as a BMI above 30 kg/m². However, in this study, we defined obesity as a BMI ≥ 25 kg/m², because of the reasons described above.

Peer review

I think it is an important paper that adds a great deal to the literature. There is not much on the topic. Your sample was large; the paper was nicely written; tables were well done and figures were very good. It was a pleasure to read.

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RAPID COMMUNICATION

Prevalence of anti-HAV antibodies in multitransfused patients with beta-thalassemia

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Abstract

AIM: To detect the prevalence of anti-HAV IgG antibodies in adult multitransfused beta-thalassemic patients.

METHODS: We studied 182 adult beta-thalassemic patients and 209 controls matched for age and sex from the same geographic area, at the same time. Anti-HAV IgG antibodies, viral markers of hepatitis B virus (HBV) and hepatitis C virus (HCV) infection were evaluated.

RESULTS: Anti-HAV IgG antibodies were detected more frequently in thalassemic patients (133/182; 73.1%) than in healthy controls (38/209; 18.2%, $P < 0.0005$). When we retrospectively evaluated the prevalence of anti-HAV IgG antibodies in 176/182 (96.7%) thalassemic patients, whose medical history was available for the previous ten years, it was found that 83 (47.2%) of them were continuously anti-HAV IgG positive, 16 (9.1%) acquired anti-HAV IgG antibody during the previous ten years, 49 (27.8%) presented anti-HAV positivity intermittently and 28 (15.9%) were anti-HAV negative continuously.

CONCLUSION: Multitransfused adult beta-thalassemic patients present higher frequency of anti-HAV IgG antibodies than normal population of the same geographic area. This difference is difficult to explain, but it can be attributed to the higher vulnerability of thalassemics to HAV infection and to passive transfer of anti-HAV antibodies by blood transfusions.

INTRODUCTION

Hepatitis A virus (HAV) hepatitis is usually spread by the fecal-oral route. Seroprevalence rates in the USA, Western Europe and in several Mediterranean countries have been falling during the past few decades. In some countries no more than 10% of the adult population has evidence of previous infection^[1]. In Greece, the lack of epidemics of HAV since early 1980s and the improvement of socioeconomic and hygienic conditions over the last decade seems to have contributed to the decline of the prevalence of anti-HAV antibodies^[2,3]. Dalekos et al tested 1984 healthy individuals of Greek nationality for anti-HAV antibodies and found a prevalence of 39.8% in males and 33.2% in females^[4]. According to the National Centre for Surveillance and Intervention of Greece, the median annual prevalence of acute hepatitis A in Greece for the time 1998-2003 was 1.88 cases/100 000 of population, which is comparable to the hepatitis A prevalence of the Western Europe^[5]. Parenteral transmission is extremely rare, but can follow transfusion of blood from a donor who is in the incubation period of the disease^[6,7]. The relatively short duration of viremia in acute hepatitis A, together with the moderate titer of HAV viral load in the blood^[8], diminishes the likelihood of transfusing a unit of blood infectious for HAV. The potential cotransfusion of HAV-specific antibodies to the recipient of multiple

blood units and the rising seroprevalence to HAV with age further diminish the risk of post-transfusion hepatitis A^[9].

The aim of this study was to detect the prevalence of anti-HAV IgG antibodies in adult multitransfused beta-thalassemic patients and to compare this with the prevalence in healthy subjects of the same age and geographic area.

MATERIALS AND METHODS

Patients

We studied 182 adult multi-transfused patients from West Peloponnese (88 males, 94 females, mean age 31.6 ± 9.4 years, range 17-66 years) suffering from beta-thalassemia major ($n = 136$) or intermedia ($n = 46$). These patients were in follow up at the Thalassemia Center of the University Hospital of Patras. They were receiving regular transfusions of two units of packed red cells at about 3 wk intervals in order to maintain the haemoglobin level above 10 g/dL. The mean age at first blood transfusion was 2 ± 1.9 years, the mean duration of transfusion therapy was 26.6 ± 8.5 years and the mean number of transfusions received up until the time of the present study was 931 ± 482 . Seventy-two patients (39.6%) had undergone splenectomy in the past. None of the patients had a history of intravenous drug use or chronic alcohol abuse. There were no reported cases of HAV hepatitis among the studied patients. In addition, none of the patients had received hepatitis A vaccination in the past. Serum used in the study was obtained just before a scheduled transfusion of packed red blood cells.

The control group was made up of 209 normal subjects from West Peloponnese, matched for age and sex (103 males, 106 females, mean age 31.2 ± 8.5 years, range 17-58 years) from the volunteer blood donor program of our Hospital. Each individual had indicated the absence of significant illness. Physical examination, normal liver function test results and absence of hepatitis B surface antigen (HBsAg) and anti-HCV antibodies in their serum, excluded liver diseases in the control group. None of the healthy subjects had a history of blood transfusion or hepatitis A vaccination in the past.

Viral markers

Anti-HAV antibodies, IgG and IgM, were tested using standard commercially available enzyme immunoassays (HAVAB-M 2.0 and HAVAB 2.0, AxSYM, Abbott Laboratories, Wiesbaden, Germany) in 182 thalassemic patients and in 209 controls from 10 February 2005 to 10 June 2005. We also retrospectively evaluated the prevalence of anti-HAV IgM and anti-HAV IgG antibodies in 176/182 (96.7%) thalassemic patients whose medical history was available for the previous ten years (about 6-8 tests for each patient). Anti-HCV antibodies were tested by third generation ELISA (AxSYM-Abbott, Wiesbaden, Germany). HCV RNA was detected in anti-HCV positive sera by reverse transcriptase polymerase chain reaction (RT-PCR) (Hepatitis C virus test-version 3.0, Cobas Amplicor, Roche Diagnostics, Branchburg, NJ, USA). Hepatitis B surface antigen (HBsAg) and hepatitis B core antibody (anti-HBc) were determined using standard 3rd

Table 1 General characteristics and prevalence of anti-HAV antibodies in beta-thalassemic patients and controls

Characteristics	Thalassemic patients (<i>n</i> = 182)	Healthy donors (<i>n</i> = 209)	<i>P</i> value
Age (yr)	31.6 ± 9.4	31.2 ± 8.5	0.662
Sex (M/F)	88/94	103/106	0.854
AST (IU/L) ¹	43.9 ± 29.1	23.9 ± 6.9	< 0.0005
ALT (IU/L)	52.6 ± 37.8	23.5 ± 10.6	< 0.0005
GGT (IU/L)	31.8 ± 26.6	22.5 ± 14	< 0.0005
Anti-HAV IgM (+) ²	0	0	
Anti-HAV IgG (+)	133 (73.1%)	38 (18.2%)	< 0.0005

¹Normal levels: Aspartate aminotransferase (AST) < 40 IU/L, alanine aminotransferase (ALT) < 40 IU/L, gamma-glutamyltranspeptidase (GGT) < 50 IU/L; ²Anti-HAV: Hepatitis A virus antibody.

generation commercially available enzyme immunoassays [AxSYM HbsAg (V2), AxSYM CORE].

Statistical analysis

Values were expressed as prevalence rates or as the mean \pm SD. Conventional chi-square and Fisher's exact test were used to analyse qualitative differences. The differences between parametric data were evaluated with Student's *t*-test. For non-parametric test values a Mann-Whitney test was used. $P < 0.05$ was considered significant. Statistical analysis was performed with SPSS 8.0 statistical software. The study was approved by the Ethical Committee of Patras University Hospital and informed consent to participate in the study was obtained from all patients and controls.

RESULTS

Prevalence of anti-HAV antibodies in the two groups

Table 1 shows the prevalence of anti-HAV antibodies in beta-thalassemic patients and controls. Anti-HAV IgM antibodies were not found in any patient of the two groups. Anti-HAV IgG antibodies were detected significantly more frequently in thalassemic patients (133/182; 73.1%) than in healthy controls (38/209; 18.2%, $P < 0.0005$). When we retrospectively evaluated anti-HAV IgM and anti-HAV IgG positivity over the past ten years in 176/182 (96.7%) thalassemic patients, we found that 83 (47.2%) of them presented anti-HAV IgG positivity continuously, 16 (9.1%) were initially anti-HAV IgG negative, but then they became persistently anti-HAV IgG positive, 49 (27.8%) presented anti-HAV IgG positivity intermittently (about two or three tests for anti-HAV were negative, while the rest of them were found positive) and 28 (15.9%) were anti-HAV IgG negative continuously. Anti-HAV IgM antibodies were consistently negative in these previous tests. It must be emphasized that the mean numbers of anti-HAV IgG tests were not significantly different between the persistently positive and the intermittent group. When we considered the intermittently anti-HAV IgG positive thalassemics as anti-HAV IgG negative we found that the difference in anti-HAV IgG positivity between thalassemics and healthy subjects was still significant (99/176; 56.3% *vs* 38/209; 18.2%, $P < 0.0005$).

Table 2 Clinical characteristics of anti-HAV positive and anti-HAV negative beta-thalassemic patients

	Anti-HAV (+) ² (n = 133)	Anti-HAV (-) (n = 49)	P value
Age (yr)	31.5 ± 9.6	31.8 ± 8.7	0.844
Sex (M/F)	62/71	26/23	0.440
Duration of transfusion therapy (y)	26.5 ± 8.1	27.1 ± 9.6	0.683
Number of transfusions	989 ± 441	772 ± 556	0.020
Splenectomy	47 (35.3%)	25 (51%)	0.055
AST (IU/L) ¹	38.6 ± 24.2	58.3 ± 36.1	0.001
ALT (IU/L)	49.2 ± 36.2	62.5 ± 40.8	0.039
GGT (IU/L)	29.6 ± 26.0	39.1 ± 27.3	0.057
Albumin (g/dL)	4.7 ± 0.45	4.47 ± 0.57	0.025
Globulins (g/dL)	3.03 ± 0.85	3.26 ± 0.79	0.122
Ferritin (median, ng/mL)	1515	1815	0.590
Anti-HCV (+)	61 (45.9%)	15 (30.6%)	0.064
HCV RNA (+)	36 (27.1%)	8 (16.3%)	0.133

¹Normal levels: Aspartate aminotransferase (AST) < 40 IU/L, alanine aminotransferase (ALT) < 40 IU/L, gamma-glutamyltranspeptidase (GGT) < 50 IU/L; ²Anti-HAV: Hepatitis A virus antibody; anti-HCV: Hepatitis C virus antibody.

Liver disease in thalassemic patients

Seventy-six thalassemic patients (41.2%) were anti-hepatitis C virus (anti-HCV) positive, while 44 (24.2%) of them were also HCV RNA positive. None of them was HBsAg positive. Anti-HBc antibody was found in 56/182 (30.8%) of them.

Comparison of anti-HAV positive and anti-HAV negative thalassemic patients

When anti-HAV positive (*n* = 133) were compared to anti-HAV negative (*n* = 49) thalassemic patients (Table 2) there was no difference in age, sex or duration of transfusion therapy. Anti-HAV positive patients had received more transfusions than anti-HAV negative thalassemics (*P* = 0.02). We also found that anti-HAV (+) thalassemics had lower frequency of previous splenectomy, although this difference did not achieve statistical significance (35.3% *vs* 51%, *P* = 0.055). An unexpected finding was that anti-HAV (+) thalassemics presented lower mean serum levels of aminotransferases (AST, *P* < 0.001; ALT, *P* = 0.039) and albumin (*P* = 0.025) than anti-HAV (-) patients. No statistical difference was found in ferritin values between these two groups. Patients with antibodies to HAV were also more frequently positive for HCV markers although this difference was not statistically significant.

DISCUSSION

Liver disease is a leading cause of death in patients with transfusion-dependent thalassemia^[10]. Transfusion-associated hepatotropic infections, especially HCV infection, and hepatic siderosis can act either synergistically or independently in promoting chronic liver disease, and they may induce cellular damage through similar oxidative pathways^[11]. There are no recent data on HAV epidemiology of that group of patients^[12-14], and blood

transfusion is not considered a significant predisposing factor for HAV infection.

In this study, adult beta-thalassemic patients were found to have significantly higher frequency of anti-HAV IgG antibodies than healthy subjects matched for age and sex in the same geographic area. IgG anti-HAV alone indicates past infection; it persists for decades after acute HAV infection and reflects recovery and immunity to reinfection^[1].

It was found that 47.2% of thalassemic patients were continuously anti-HAV (+) over the past ten years. The possibility that multitransfused patients with beta-thalassemia acquire anti-HAV antibodies with higher frequency than normal people of the same geographic area because of socioeconomic reasons is difficult to be supported. There are also no data demonstrating a higher susceptibility of patients with thalassemia for hepatitis A. Unfortunately, we did not study the prevalence of anti-HAV antibodies in family members of these thalassemia patients. Immune deficiency attributed to multiple transfusions and to iron overload^[15] could be a factor that could predispose thalassemics to HAV infection, but no data exist that immune deficiency per se predispose to HAV infection. However, 41.2% of thalassemics were anti-HCV positive and 24.2% of them were HCV-RNA positive. In addition, thalassemics with anti-HAV positivity presented higher percentage of anti-HCV and HCV-RNA positivity, although these differences were not statistical significant. Two studies from Italy have shown that patients with chronic liver disease present high seropositivity for anti-HAV^[16,17].

Another interesting finding was that 9.1% of thalassemic patients acquired anti-HAV IgG antibody during the previous ten years. However, anti-HAV IgM antibodies were not found in these patients during this time, so it is difficult to prove acute infections. IgM anti-HAV in serum is positive from the onset of symptoms and usually remains positive for approximately 4 mo^[18]. These tests were performed every 1-2 years and it is possible not to detect IgM anti-HAV antibodies in these patients. This is a high rate of HAV seroconversion even for countries of high endemicity. Greece is considered, according to studies of previous decade as a country of low endemicity for HAV infection, with some regions of intermediate endemicity^[2-5]. Transfusion-associated hepatitis A virus is possible, but it is a very rare event (one per million transfusions)^[19-22], so there is no evidence to consider polytransfused patients as a risk group for parenteral hepatitis A infection. Due to the frequent stay of thalassemics in ambulant and hospital premises of the health care system, hospital borne hepatitis A infection should also be taken into consideration. However, the transmission from the medical care stuff to the patients is even more rare^[23]. In general, hospital-borne hepatitis A disease does not seem a possible explanation of HAV infection in thalassemics.

Finally, 27.8% thalassemics presented anti-HAV positivity intermittently. In this case anti-HAV IgG is apparently not the expression of an actively acquired immunity, but might have been transferred passively

by means of transfusions. It is well known that packed red cells contain leukocytes, microaggregates, plasma containing proteins and immunoglobulins^[24]. The level of protection that might be conferred by these antibodies is unknown. This finding is similar to that in substituted haemophiliacs where the detection of anti-HAV antibodies is also a frequent finding^[25,26]. In hemophiliacs, the high concentration of antibodies in plasma preparations of multiple donors and the frequency of their administration facilitate the passive transmission of antibodies. It can be supported that some of the continuously anti-HAV (+) sera in the other thalassemic patients could be also attributed to the passive transfer of anti-HAV antibodies. We think that it is not very possible, as many tests (6-8) for anti-HAV antibodies just before a transfusion and for the previous ten years were found persistently positive.

Previous studies have shown lower prevalence of anti-HAV antibodies in multiply transfused thalassemic patients^[12-14]. However, in these studies the mean age of thalassemic patients was much lower than that of our population and it is known that seroprevalence rates of HAV are increasing with age.

The lower mean levels of AST in anti-HAV IgG positive thalassemics, is difficult to explain. Ferritin values were found comparable between the two groups. Body mass index (BMI) and alcohol consumption were not different between these two groups. Moreover, no statistical difference was found in anti-HCV or HCV RNA positivity between anti-HAV positive and anti-HAV negative thalassemics that could explain this difference between the two groups.

There are some important limitations in this study. Volunteer blood donors are selected subjects, not comparable with general population. In cases where passive transfer of anti-HAV antibodies was suspected, the frozen aliquots of the donor's plasma were not tested for the presence of anti-HAV antibodies. Additional studies may be needed to confirm our findings.

In accordance to the findings of this study, we suggest that thalassemic patients present higher prevalence of anti-HAV IgG antibodies than matched healthy subjects of the same geographic area. This difference is difficult to explain, but it can be attributed to the higher vulnerability of thalassemics to HAV infection and to passive transfer of anti-HAV antibodies by blood transfusions.

COMMENTS

Background

In Greece, the lack of epidemics of hepatitis A virus (HAV) since early 1980s and the improvement of socioeconomic and hygienic conditions over the last decade seems to have contributed to the decline of prevalence of anti-HAV antibodies. However, we observed that the majority of our beta-thalassemia patients were anti-HAV positive.

Innovations and breakthroughs

Previous studies have shown lower prevalence of anti-HAV antibodies in multiply transfused thalassemic patients. However, in these studies the mean age of thalassemic patients was much lower than that of our population and it is known that seroprevalence rates of HAV are increasing with age.

Applications

In order to reduce further the incidence of liver infections in polytransfused thalassemic patients, we recommend an active immunization for HAV.

Terminology

Beta-thalassemia, also known as Cooley's anemia, is a chronic recessively inherited hemoglobinopathy, characterized by severe hemolysis.

Peer review

The authors reported that multitransfused adult beta-thalassemic patients present higher frequency of anti-HAV IgG antibodies than normal population of the same geographic area. This result is interesting. The strength of this study is surely the dimension of the population and the long history of the patients (10 years).

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RAPID COMMUNICATION

Prevalence and determinants of delayed gastric emptying in hospitalised Type 2 diabetic patients

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associated with higher probability of delayed GE.

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Key words: Autonomic neuropathy; Diabetes mellitus; Gastric emptying; Gastrointestinal symptoms; Glycemic control

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Abstract

AIM: To determine the prevalence of delayed gastric emptying (GE) in older patients with Type 2 diabetes mellitus.

METHODS: One hundred and forty seven patients with Type 2 diabetes, of whom 140 had been hospitalised, mean age 62.3 ± 8.0 years, HbA1c $9.1\% \pm 1.9\%$, treated with either oral hypoglycemic drugs or insulin were studied. GE of a solid meal (scintigraphy), autonomic nerve function, upper gastrointestinal symptoms, acute and chronic glycemic control were evaluated. Gastric emptying results were compared to a control range of hospitalised patients who did not have diabetes.

RESULTS: Gastric emptying was delayed ($T_{50} > 85$ min) in 17.7% patients. Mean gastric emptying was slower in females ($T_{50} 72.1 \pm 72.1$ min vs 56.9 ± 68.1 min, $P = 0.02$) and in those reporting nausea (112.3 ± 67.3 vs 62.7 ± 70.0 min, $P < 0.01$) and early satiety (114.0 ± 135.2 vs 61.1 ± 62.6 min, $P = 0.02$). There was no correlation between GE with age, body weight, duration of diabetes, neuropathy, current glycemia or the total score for upper gastrointestinal symptoms.

CONCLUSION: Prolonged GE occurs in about 20% of hospitalised elderly patients with Type 2 diabetes when compared to hospitalised patients who do not have diabetes. Female gender, nausea and early satiety are

INTRODUCTION

It is now recognised, albeit relatively recently, that disturbances in upper gastrointestinal motility represent a major cause of morbidity in diabetic patients^[1] and affect quality of life adversely^[2]. Delayed gastric emptying is arguably the most important gastrointestinal complication and has been reported to affect 30%-60% of patients with long-standing Type 1 diabetes^[3]. In Type 1 patients gastric emptying (GE) is occasionally faster than in normal during euglycemia^[4]. There is less information about the prevalence of gastroparesis in Type 2 diabetes^[5,6] and the majority of the cohorts studied have been small^[2,7]. There is no information about the prevalence of delayed gastric emptying in older patients with diabetes Type 2 who are in many respects different from Type 1 patients. In Type 1 patients it is recognised that the rate of gastric emptying is modulated by acute changes in blood glucose concentration (gastric emptying is slower during hyperglycemia^[8,9] and accelerated during hypoglycemia^[10]) and autonomic nerve function. The relationship between gastroparesis and autonomic dysfunction is, however, relatively weak^[11,12]. The prevalence of gastroparesis is also dependent on the duration of diabetes and may be higher in females than males^[13]. The relationship of upper gastrointestinal symptoms with the rate of gastric emptying in Type 1 patients is poor^[11].

The aims of the study were to evaluate the prevalence,

and determinants, of disordered gastric emptying in a large cohort of predominantly hospitalised, Type 2 patients and the relationship of upper gastrointestinal symptoms with gastric emptying. The latter was quantified using a scintigraphic technique, which is considered as the “gold standard”^[5,14-16].

MATERIALS AND METHODS

The study was performed in 147 patients with Type 2 diabetes (76 women, 71 men), mean age 62.3 ± 8.0 years, duration of known diabetes 11.4 ± 7.2 years, BMI 31.2 ± 5.5 kg/m². One hundred and twenty four patients (84%) had BMI > 25. 72 of the patients (49%) were managed on oral hypoglycemic therapy alone (sulphonylurea and/or metformin, thiazolidindione) and the remaining 75 patients were on insulin. None was on combination of insulin and oral drugs. All but 7 of the 147 patients were hospitalised in patients who were randomly selected from those admitted to the Internal Medicine Department. The primary reasons for hospitalisation are summarised in Table 1.

Subjects were randomly selected from among all patients with Type 2 diabetes treated with hypoglycemic drugs or insulin and without exclusion criteria. Only one patient was enrolled each day because of limitations in the availability of scintigraphy. Exclusion criteria included previous gastrointestinal disease apart from uncomplicated appendectomy, symptomatic cardiac, hepatic, renal or pancreatic disease, the use of medication known to affect gastrointestinal motility^[17-19] and acute derangement of diabetes during the preceding week. Studies were performed on the day after hospital admission.

Protocol

In one day patient underwent assessments of upper gastrointestinal symptoms, gastric emptying, glycemic control and autonomic nerve function. An assessment of diabetic retinopathy was performed within one week. The subjects were studied after an overnight fast and were asked to take their usual dose of insulin or oral hypoglycemic medication about 30 min prior to ingestion of the test meal.

Capillary blood samples for the measurement of blood glucose (enzymatic-amperometric measurement EBIO Plus) were obtained immediately before the meal and at 30, 60, 90 and 120 min. Glycated hemoglobin was measured on one of the blood samples. Each patient also completed a questionnaire relating to upper gastrointestinal symptoms^[11]. Ophthalmological examination was performed to assess diabetic retinopathy, unless this diagnosis had already been established.

Autonomic nerve function was assessed after the completion of gastric emptying measurement using power spectral analysis of heart rate variability. The electrocardiogram was recorded under standardised conditions^[20] for 5 min (minimum 300 valid R-R intervals) in supine, standing and repeated supine positions using computer-based system VarCor PF6^[21]. Spectral analysis using fast Fourier transformation was performed in very low (0.02-0.05 Hz), low (0.05-0.15 Hz) and high (0.15-0.5 Hz) frequency spectral bands. The sum of the overall spectral power in the high and low fre-

Table 1 Reasons for hospitalisation in type 2 diabetic (*n* = 147) and nondiabetic (*n* = 34) patients

	Diabetes mellitus <i>n</i>	Control group <i>n</i>
Diabetes mellitus	80	-
Hypertension	21	12
Ischemic heart disease	16	4
Back pain	6	4
Valvular heart disease	4	-
Colonic polyp	3	-
Anemia	2	-
Peripheral atherosclerosis	2	1
Gout	2	-
Phlebitis	1	3
Chronic bronchitis	1	1
Nontoxic goiter	1	-
Arthrosis	1	1
Atrial fibrillation	-	3
Neurasthenia	-	3
Pacemaker implantation	-	2

quency part of the spectra with patients in all relevant positions and the sum of the spectral power in the low frequency part of the spectra were calculated^[22,23]. Values obtained in healthy subjects were used as a standard^[22]. Spectral power values less than normal (after adjustment for age) were considered as evidence of cardiovascular autonomic neuropathy (CAN). For the purpose of statistical assessment both borderline and abnormal results were combined.

Upper gastrointestinal symptoms during the previous 3 mo were assessed using a questionnaire adopted from Horowitz *et al*^[11]. Anorexia, nausea, early satiety, vomiting, abdominal pain, bloating and heartburn were recorded and graded as 0 = none, 1 = mild, 2 = moderate, 3 = severe. The global symptom score was calculated as the sum of the number and severity of symptoms.

Written informed consent was obtained from all subjects. Data were evaluated using the Mann-Whitney test, Kruskal-Wallis ANOVA and Spearman correlation, *P* values < 0.05 were considered as significant.

Measurement of gastric emptying

The test meal consisted of 100 g of boiled rice (560 kJ) flavoured with cinnamon and labelled with 185 mBq 99^mTc sulphur colloid^[24,25]. The study was performed in the decubitus position and data were acquired for at least 60 min, in 1 min frames using a Picker Int. one-head camera. Time zero was defined as the time of a meal completion. Data were corrected for radionuclide decay and analysed using the Picker Int. Odyssey computer system. The gastric half-emptying time (T50) was defined as the time for the activity to fall by 50%. In those subjects in whom total gastric activity at the end of investigation was greater than 50% this parameter was computed using software extrapolation. The lag phase was defined as the period from meal ingestion to the peak of the activity within the gastric region of interest.

Control values were determined from a group of 34 nondiabetic volunteers. Nineteen women, 15 men, mean age 60.8 ± 8.9 years, mean BMI 30.7 ± 4.7 kg/m², 27 subjects

(79%) had BMI > 25. This control group was randomly selected from among hospitalised patients without diabetes and without dyspeptic symptoms using identical exclusion criteria. Reasons for their admission are listed in Table 1. In this group mean T50 was 49.5 ± 23.1 min and lag phase 7.1 ± 5.9 min. The normal range for gastric emptying was defined as mean ± 1.5 SD of the T50. Values outside of this were considered as abnormal.

RESULTS

All of the studies were well tolerated. Mean glycated hemoglobin was $9.1\% \pm 1.9\%$. Abnormal autonomic nerve function was evident in 103 of 144 (71.5%) patients (in 3 subjects the assessment was not feasible due to their high age or technical artefacts). Diabetic retinopathy (nonproliferative and proliferative) was evident in 21 (14.3%) cases. 52 patients had upper gastrointestinal symptoms and symptoms were reported more frequently by women (46.0% *vs* 23.9%, $P < 0.01$). The most frequent symptom was bloating (29 subjects), followed by early satiety (10 subjects), pain (8 subjects), nausea and anorexia (6 subjects), heartburn (5 subjects), and vomiting (4 subjects). The median symptom score was 0 (0-9) in the whole group, 2.0 (1-9) in subjects with normal emptying and 3.0 (1-5) in the subgroup with delayed gastric emptying.

The mean T50 was 64.7 ± 70.4 min, and lag phase 9.6 ± 7.9 min, these were related ($P < 0.01$). Gastric emptying was delayed, as assessed by the T50, in 26 subjects (17.7%) and in the whole group was significantly prolonged comparing to the control group (64.7 ± 70.3 min, 49.5 ± 23.4 min, $P = 0.03$). Mean basal (time 0) glucose was 9.9 ± 2.8 mmol/L, at 30 min 10.5 ± 2.8 mmol/L, at 60 min 11.5 ± 2.9 mmol/L, at 90 min 11.7 ± 3.1 mmol/L, and at 120 min 11.6 ± 3.2 mmol/L. The mean rise in blood glucose was significant ($P < 0.01$). The association of gastric emptying with demographic and biochemical variables and gastrointestinal symptoms is summarised in Table 2.

Gastric emptying did not differ between insulin and oral drug treated subjects (68.4 ± 83.7 min, 60.4 ± 50.9 min, $P = 0.47$). Gastric emptying (T50) was slower in females than males ($P = 0.02$). Gastric emptying (T50) was not significantly related to either autonomic nerve function ($P = 0.32$), retinopathy ($P = 0.88$) or upper gastrointestinal symptoms ($P = 0.22$). Gastric emptying was however, slower in those patients who had early satiety ($P = 0.02$) and nausea ($P < 0.01$).

Simple regression analysis showed a weak, but significant, relationship between gastric emptying and glycated hemoglobin ($r = 0.23$; $P = 0.01$), but not between the T50 ($P > 0.35$) or lag phase ($P > 0.07$) and blood glucose levels during the gastric emptying measurement ($P > 0.35$). Significant correlation between gastric emptying rate and glycated hemoglobin was noted in the subgroup treated with hypoglycemic drugs ($r = 0.32$; $P < 0.01$). There was no significant relationship between gastric emptying and either age ($r = -0.05$, $P = 0.55$), duration of diabetes ($r = 0.08$, $P = 0.31$) or body mass index ($r = -0.03$, $P = 0.70$). GE did not differ between obese and non-obese subjects both in the diabetes (66.9 ± 74.6 min, 50.6 ± 28.4 min, $P = 0.16$) and in the control

Table 2 Association between demographic variables and gastric emptying in Type 2 patients

	<i>n</i>	T50 (min)			Lag phase (min)		
		mean	1 SD	<i>P</i>	mean	1 SD	<i>P</i>
Gender				0.02 ^a			0.99
Female	76	72.1	72.1		9.1	6.4	
Male	71	56.9	68.1		10.1	9.3	
CAN				0.32			0.66
No	41	64.1	84.4		10.2	8	
Yes	103	65.6	65.3		9.6	8	
Retinopathy				0.88			0.77
No	126	65.7	74.4		9.8	8.2	
Yes	21	58.7	38.9		8.5	6.1	

CAN: Cardiovascular autonomic neuropathy. ^a $P < 0.05$ between male and female.

group (48.6 ± 23.4 min, 56.0 ± 24.8 min, $P = 0.25$).

In the insulin treated group (75 subjects) the rise of glycemia between 30-60 min. (16.2% *vs* 5.8%) was greater ($P = 0.048$) in subjects with faster emptying compared to those with delayed GE. In the group treated with oral hypoglycemic drugs the changes were not significantly different.

DISCUSSION

This is the first study to evaluate the prevalence of delayed gastric emptying in a large cohort of elderly predominantly hospitalised patients with Type 2 diabetes. We have established that (1) when compared to a control range obtained in hospitalised patients without diabetes prolonged GE does not occur frequently and (2) female gender and the presence of nausea and early satiety are associated with higher probability of delayed GE.

Delayed GE is said to occur in 30%-70% patients with longstanding Type 2 diabetes^[5,7]. Some of the cohorts studied were small, e.g. Tung studied 20 subjects with diabetes^[7], and Annese *et al* investigated 35 subjects^[2], included otherwise healthy patients with diabetes^[5], or patients with upper gastrointestinal symptoms^[7], which may not well reflect the situation in general population. We intentionally studied a large cohort of relatively older patients with more severe Type 2 diabetes treated with oral drugs or insulin, of whom the majority had complicated diabetes, and compared them with their age-matched non-diabetic counterparts. In our opinion, this subset of patients corresponds with the status of the older general population. The prevalence of delayed gastric emptying was relatively low and less than we anticipated. Any correlation of our results with other studies is rather difficult due to substantial differences in patient groups and methods used^[5]. Alteration of the results due to test food used, however, appears unlikely, given that the mean rate of gastric emptying in the diabetic cohort (8.7 kJ/min) was within the expected "physiological" range (i.e. 8.4 to 12.6 kJ/min)^[1,26]. This confirms that test food used by us is sufficient for activation of feedback regulation, which physiologically slows the emptying down. In a previous study, we found that GE was delayed in 42.8% of Type 1 diabetic outpatients^[27] us-

ing the same method, and identical control group, a result that is concordant with previous reports^[4,14,25]. Also, it is against any possible bias caused by technical errors.

Our group involved a selected set of hospitalised subjects where a selection bias may play a role. Any effect of comorbidities on GE has not been so far sufficiently studied. There are some indices that there may exist a certain link between gastric emptying rate and heart, lung, liver diseases or Parkinson's disease^[28], occurring frequently in older age. More than 80% of patients screened in this study were treated with other than oral hypoglycemic drugs. Information on the effect of commonly used drugs like beta-blockers, calcium channel blockers, etc. on GE is lacking. Only verapamil and nitrates have been studied so far^[29]. The accelerated GE was described in obese patients, e.g. by Bertin^[30] and was also documented in obese non-diabetic persons^[31]. In spite of the fact that weight in our group was similar to that in Bertin's group, we were not able to confirm any correlation with GE.

There was no significant relationship between the rate of GE and the duration of known diabetes, consistently with previous reports in Type 1 and groups of Type 1 and 2 diabetic patients^[6,32]. While, the number of complications increases with the duration of diabetes, their development is highly dependent on glycemic control. This is likely to confound a potential association with the duration of diabetes. In the past autonomic neuropathy was considered to be the main factor causing gastroparesis, but it is clear that any relationship between gastric emptying and cardiovascular autonomic function is weak^[5,12,33]. The prevalence of autonomic nerve dysfunction observed in this study is higher than that reported in groups with a similar duration of diabetes (about 65% after 10 years)^[34] what is likely to reflect the methodology used. Furthermore, heart rate variability and spectral power is known to decline not only in individuals with diabetic CAN but also in those with ischemic heart disease, poor physical status and/or ageing^[23]. It is well known that the presence of diabetic retinopathy correlates with that of CAN^[35]. Both retinopathy and CAN are associated with higher mortality while gastroparesis may not be^[7,36,37]. Female gender has been reported previously^[13] to be associated with an increased rate of diabetic gastroparesis; this is also the case for delayed gastric emptying in functional dyspepsia^[38]. The underlying cause(s) remain uncertain.

Acute glycemia has been suggested as an important determinant of gastric emptying^[14,39]. However, there are studies^[25,30], including the present one, which have failed to confirm this relationship. A possible explanation is, that in spite of relatively large variations in glycemia during the studies (3.1–22.3 mmol/L), average values were about 11 mmol/L, and only 10 individuals had blood glucose levels above 15 mmol/L. The present study is also cross-sectional in design, which is not optimal for evaluation of this issue; furthermore, there was a weak relationship between the rate of gastric emptying with HbA1c.

It is clear that the GE rate of carbohydrate influences postprandial glycemia in both Type 1 and Type 2 diabetes^[40]—in Type 2 patients the postprandial initial glycemic and insulinemic responses to oral carbohydrate are less when gastric emptying is slower^[41]. Hence it is not surprising that the rise in blood glucose after the meal

was greater in those with faster gastric emptying in those patients treated with insulin. In contrast there is no difference apparent in the patients treated with oral hypoglycemic agents for which we have no clear explanation.

Previously it was thought that dyspeptic symptoms associated with diabetes were a direct consequence of impaired GE, but the correlation between upper gastrointestinal symptoms and the rate of GE rate is weak^[5,11,25] and patients with gastroparesis may not have symptoms at all^[3]. Our study confirms that poor correlation between gastrointestinal symptoms and gastric emptying described in Type 1 diabetic patients^[4,27] also exists in Type 2 diabetes. Recent studies have found an association between abdominal bloating and fullness, particularly postprandially^[6,36,42] and slow GE, especially when the relationship is assessed according to the severity of symptoms. This study confirms that correlations observed between distinct gastrointestinal symptoms (early satiety/fullness) and GE in Type 1 diabetes^[4] may also exist in Type 2 diabetes^[6]. These relationships should be, however, interpreted with caution and in the context of other variables. For example, postprandial dyspeptic complaints may potentially result from deranged intragastric distribution of ingested food, which however, may not be associated with any in the total GE rate^[43]. It is also well established that blood glucose concentrations may modulate sensations arising from the gastrointestinal tract^[44]. The authors were not able to confirm any such dependence; nevertheless mean blood glucose levels were not as high as those reported by Jones *et al* (> 15 mmol/L) in their study^[45], where the relationship was described.

In conclusion, the present study demonstrates, that delayed GE rate does not occur frequently in elderly hospitalised Type 2 diabetics. Female gender and the presence of nausea and early satiety may predict delayed gastric emptying. Autonomic nerve dysfunction, assessed by means of heart rate variability, however does not correlate with GE rate.

COMMENTS

Background

Delayed gastric emptying is the most important gastrointestinal complication in diabetes. The problem of finding a simple way of identifying persons with gastroparesis is gaining ground in view of the rising proportion of Type 2 diabetic patients. An attractive approach appears to identify groups at risk appropriate for further detailed investigation by using simple criteria. This issue has been studied extensively in Type 1 diabetic patients. In contrast, data have been lacking and study groups are small in patients with diabetes Type 2, who are in many respects different from Type 1 patients.

Research frontiers

To evaluate the prevalence, and determinants (gender, age, BMI, duration of diabetes, control of diabetes, autonomic neuropathy, retinopathy and dyspeptic symptoms) of delayed gastric emptying in older patients with Type 2 diabetes mellitus.

Innovations and breakthroughs

This is the first study evaluating elderly Type 2 diabetic patients with comorbidities. Delayed GE rate does not occur frequently in these subjects. They do not differ in predictors of delayed GE from Type 1 diabetes.

Applications

Delayed gastric emptying in old Type 2 diabetic patients may be assumed in

female gender and when nausea and early satiety are present. Presence of diabetic complication (retinopathy, autonomic neuropathy) does not predict disordered gastric emptying.

Peer review

This study explores the presence of dyspeptic symptoms and the timing of gastric emptying in hospitalised elderly patients with Type 2 diabetes treated with oral hypoglycaemic therapy or insulin, compared to hospitalised patients without diabetes. The study is well designed and conducted.

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RAPID COMMUNICATION

Mutations in components of the Wnt signaling pathway in gastric cancer

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Abstract

AIM: To explore the contribution of AXIN1, AXIN2 and beta-catenin, components of Wnt signaling pathway, to the carcinogenesis of gastric cancer (GC), we examined AXIN1, AXIN2 exon7 and CTNNB1 (encoding beta-catenin) exon3 mutations in 70 GCs.

METHODS: The presence of mutations was identified by polymerase chain reaction (PCR)-based denaturing high-performance liquid chromatography and direct DNA sequencing. Beta-catenin expression was detected by immunohistochemical analysis.

RESULTS: Among the 70 GCs, 5 (7.1%) had mutations in one or two of these three components. A frameshift mutation (1 bp deletion) in exon7 of AXIN2 was found in one case. Four cases, including the case with a mutation in AXIN2, had frameshift mutations and missense mutations in AXIN1. Five single nucleotide polymorphisms (SNPs), 334 C>T, 874 C>T, 1396 G>A, 1690 C>T and 1942 T>G, were identified in AXIN1. A frameshift mutation (27 bp deletion) spanning exon3 of CTNNB1 was observed in one case. All four cases with mutations in AXIN1 and AXIN2 showed nuclear beta-catenin expression.

CONCLUSION: These data indicate that the mutations

in AXIN1 and AXIN2 may contribute to gastric carcinogenesis.

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Key words: AXIN1; AXIN2; β -catenin; Wnt signaling pathway; Gastric cancer

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INTRODUCTION

The Wnt signaling pathway plays an essential role in human cancer development^[1-3]. Wnt stabilizes cytoplasmic β -catenin, which stimulates the expression of genes including c-myc, c-jun, fra-1 and cyclin D1^[4,5]. AXIN1 and its homologue AXIN2, newly recognized as components of the Wnt signaling pathway, negatively regulate this pathway^[6,7]. Other components of the Wnt signaling pathway, including β -catenin and adenomatous polyposis coli (APC), interact with AXIN, and the phosphorylation and stability of β -catenin are regulated in the AXIN complex^[8].

AXIN1 is thought to be critical for degrading cytoplasmic β -catenin. Different domains of AXIN1 were shown to interact with APC, GSK-3 β , β -catenin, PP2Ac and AXIN itself^[8,9]. The function of AXIN1 in promoting β -catenin degradation suggests that it serves as a tumor suppressor. AXIN1 mutations have been reported in a variety of human carcinomas including sporadic medulloblastomas, hepatocellular carcinomas and colorectal cancers^[10-13], suggesting that AXIN1 may be involved in the development of these tumors.

AXIN2, which functions as a scaffold protein in the Wnt signaling pathway^[14], has been shown to develop frameshift mutations in mononucleotide repeat sequences located in exon7, in colorectal cancer, with defective DNA mismatch repair (MMR)^[7]. A recent study has shown that AXIN2 is a transcriptional target of the TCF/LEE transcription factor complex downstream of activated β -catenin^[15].

Mutations in CTNNB1 (encoding β -catenin) or APC have been reported in human neoplasms, including colon cancer and gastric cancer (GC)^[16-19]. However, AXIN1 and AXIN2, the key players in this pathway, have not been investigated in GC. In this study, we identified mutations of the entire coding region of AXIN1, exon7 of AXIN2 (a hot spot of mutation), and exon3 of CTNNB1 in GC cases, and evaluated if such mutations activate the Wnt signaling pathway during GC development.

MATERIALS AND METHODS

Tissue specimens and DNA isolation

GC samples and their matched normal gastric tissues were obtained from 41 males and 29 females during surgical resections. The study was approved by Institutional Review Board of Peking University School of Oncology, and all subjects gave written informed consent. Fresh samples were collected at the time of surgery, frozen in liquid nitrogen and stored at -80°C . Sections from each specimen were determined by a pathologist, and classified according to Lauren's classification^[20]. Among the GC cases, 32 (46%) were intestinal-type, 27 (39%) were diffuse-type and 11 (16%) were mixed-type. High molecular weight genomic DNA was extracted by standard proteinase K digestion and phenol/chloroform extraction.

Primers and PCR

We screened the entire coding sequence of the AXIN1 gene, exon7 of AXIN2 and exon3 of CTNNB1 with PCR-based denaturing high-performance liquid chromatography (DHPLC). The primer sequences are listed in Table 1. PCR was accomplished with a 25 μL reaction mixture containing 50 ng of genomic DNA, 0.4 $\mu\text{mol/L}$ sense and antisense primers for each exon, 200 $\mu\text{mol/L}$ dNTPs (Perkin-Elmer, CA, USA), 0.2 μL of Ampli Taq Gold polymerase (Perkin-Elmer, USA), and 2.0 mmol/L of MgCl_2 . After initial activation of the enzyme by denaturation at 95°C for 9 min, PCR amplification was performed for 35 cycles: 94°C for 30 s, the optimized annealing temperature for 45 s, and 72°C for 45 s. Final extension was performed at 72°C for 10 min. The annealing temperatures for various primer sets were: 58°C for AXIN1 exons 1A, 1B, 1C, 2, 3, 4 and 9; 63°C for exons 5A, 6, 7, 8 and 10; 62°C for exon 5B; 60°C for P3-4; and 59°C for AXIN2 exon 7.

DHPLC analysis

PCR products were heated to 95°C for 3 min, then cooled to 25°C over 45 min. Homozygous mutant DNA was combined with wild-type in an approximately 1:1 ratio prior to hybridization; then, this mixture was examined for heteroduplex content by subjecting 50-100 ng of PCR products to DHPLC (WAVETM system, Transgenomic, USA) under partial denaturation conditions^[21,22]. The mobile phase consisted of a mixture of 0.1 mol/L triethylamine acetate (TEAA, pH 7.0) with or without 25% acetonitrile. The flow rate used in this study was 0.9 mL/min. The column temperatures for various PCR products were: 59°C for P3-4; 62°C for AXIN1 exons 1A, 1B, 1C and 3; 63°C for exons 2 and 10; 64°C for exons 7 and 9; 65°C for exons 5A

and 6; 66°C for exons 5B and 8; 68°C for exon 4; and 66°C for AXIN2 exon 7.

Sequencing analysis

PCR products were treated with exonuclease and shrimp alkaline phosphatase based on the protocol provided by United States Biochemical Corporation and sequenced by the Mayo Clinic DNA sequencing facility. Sequencing reactions were performed in a GeneAmp PCR System 9600 with fluorescent terminations, and products were analyzed on an ABI 377 sequencer (Perkin-Elmer). All sequence alterations were confirmed by bidirectional sequencing of PCR products generated by at least two independent reactions.

Immunohistochemical staining

Tissue specimens were fixed in 10% neutral-buffered formalin and were paraffin embedded according to standard procedures. Four-micrometer-thick sections of representative blocks from each case were deparaffinized in xylene, rehydrated, and treated with 3% H_2O_2 for 10 min to block endogenous peroxidase activity. All sections were subjected to heat-induced epitope retrieval in a microwave oven. Sections were incubated with anti- β -catenin mouse monoclonal antibody (1:50, Santa Cruz, USA) at 4°C overnight. The sections were treated with polyperoxidase anti-mouse/rabbit immunoglobulin (GBI) for 30 min at 37°C and antibody-binding sites were visualized by DAB kit (Zhongshan Golden Bridge Co., Beijing, China). Negative controls were performed by omitting the primary antibody.

RESULTS

We evaluated the entire coding region of the AXIN1 gene for mutations by DHPLC followed by sequencing. Two types of mutation were identified in 4 (5.7%) of 70 GC cases, but not in the matched normal tissues. Three cases, including one intestinal-type and two diffuse-type, contained a 1-bp deletion at nucleotide 1076 (Figure 1A). This mutation interrupts the GSK3 β and β -catenin binding domains. A point mutation of G to T transition at nucleotide 1578 was observed in one intestinal-type, resulting in a substitution of the amino acid at 489 from glycine to valine (Figure 2). This missense mutation occurs in the β -catenin binding domain. Five single-nucleotide polymorphisms, 334 C>T, 874 C>T, 1396 G>A, 1690 C>T and 1942 T>G, were identified within the coding region. The frequencies of these variant alleles were 0.04, 0.43, 0.10, 0.03 and 0.13, respectively (Table 2). All of them were silent polymorphisms, and the functions of these polymorphisms are unknown. Representative examples of heteroduplexes detected by DHPLC are shown in Figure 1B.

We screened exon7 of the AXIN2 gene for mutations and one intestinal-type revealed a frameshift mutation (1 bp deletion), occurring in the mononucleotide repeat sequences (2083 del G) located in exon7 (Figure 1C). This mutation leads to the elimination of the DIX domain. This case had AXIN2 and AXIN1 (1 bp deletion at nucleotide 1076) mutations.

Table 1 Primers used for AXIN1, AXIN2 and CTNNB1 gene amplification

Exon	Sense primer	Antisense primer
AXIN1		
exon1A	5'-TGGTCGIGTTTCATGGACCC-3'	5'-AATGCAGTGACTCAGCCCACT-3'
exon1B	5'-GGATCTGGACCTGGGTATG-3'	5'-ATAGTGGCTGGATTTCGGT-3'
exon1C	5'-ATCATGAAGCAGCTGATCG-3'	5'-GAGGTGAGTACAGAAAGTGGAC-3'
exon2	5'-CTGTTGGCAGGCTGTCCT-3'	5'-GTCCGTGAGGGACTGGTA-3'
exon3	5'-CTGGCCCTCCTGCTCTCT-3'	5'-AGGACGATGGGCTGAGGAC-3'
exon4	5'-TTTAGCCTGTGACCTTTCAAC-3'	5'-ATCCCGGCGGCAAGAA-3'
exon5A	5'-AGCTGGTGTGAGAGGTGATG-3'	5'-CCCTGACTTGGGTACGTGCTT-3'
exon5B	5'-TGGGCACGTGGCCAAGAT-3'	5'-AAGCCCCCTCCTCACTGACAG-3'
exon6	5'-CACCGAGGCCAGGGCGACT-3'	5'-TGGCAAAGCAGGCCCCACGA-3'
exon7	5'-CCAGGGTGTGCGCCACAGTC-3'	5'-CCCCAGGAGTGGTGTGCTGGT-3'
exon8	5'-GCGCAGCAGCATTTGTCGAG-3'	5'-GGGCAGGACCGGGAGGACC-3'
exon9	5'-AGTCCAAAACCAGGTACCAC-3'	5'-AAACCTCTTTTCATACCG-3'
exon10	5'-CACGCCGTCCCCTGCCAC-3'	5'-GACACCCGTGCCCGCCAA-3'
AXIN2		
exon7	5'-TCCTTGTTTGGCCAAAGC-3'	5'-GGTCAGGGAGGCATCGCAG-3'
CTNNB1		
exon3		
P3-4	5'-GATTGATGGAGTTGGACATGG-3'	5'-TGTTCTTGAGTGAAGGACTGAG-3'

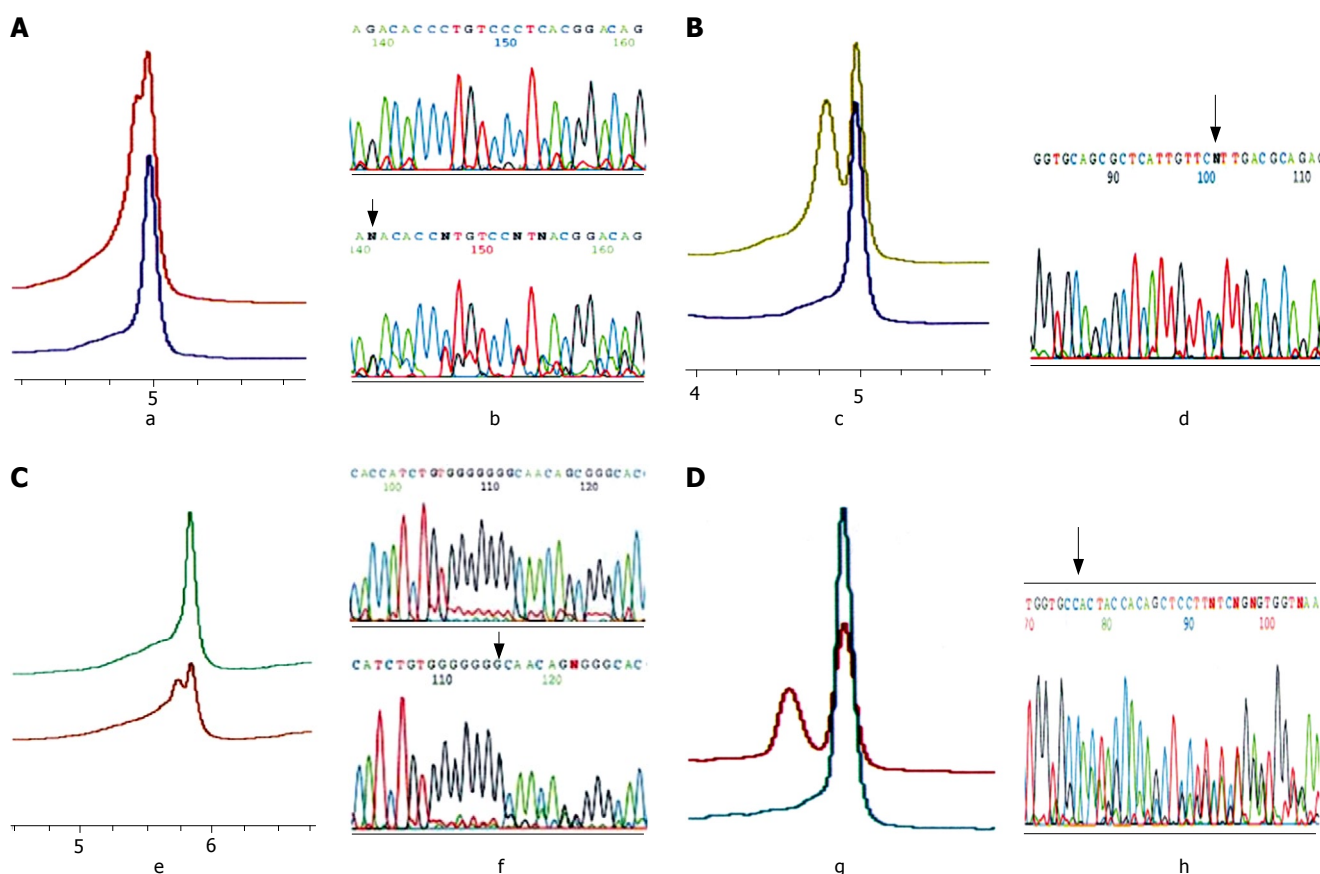


Figure 1 Typical elution profiles of DHPLC analysis and sequencing. **A:** AXIN1 (exon2) mutation. **a,** DHPLC elution profiles. **b,** Sequence traces. The upper panel is a normal control; the lower panel is a frameshift mutation (del 1 bp). The arrow points to the mutant nucleotide; **B:** AXIN1 polymorphism. **c,** Representative DHPLC profiles of heteroduplex for AXIN1. The lower panel is a normal control; the upper panel is a heteroduplex. **d,** Sequence traces. The arrow points to the mutant nucleotide; **C:** AXIN2 (exon7) mutation. **e,** DHPLC elution profiles. **f,** Sequence traces. The upper panel is a normal control; the lower panel is a frameshift mutation (del 1 bp). The arrow points to the mutant nucleotide; **D:** CTNNB1 (exon3) mutation. **g,** DHPLC elution profiles. The lower panel is a normal control; the upper panel is a frameshift mutation (del 27 bp spanning exon3). **h,** Sequence traces for the frameshift mutation. The arrow points to the mutant nucleotide.

Somatic mutations in exon3 of the CTNNB1 gene were detected in the same samples. A 27-bp deletion spanning exon3 of CTNNB1 was observed in one of the diffuse-type cases (Figure 1D), which did not contain AXIN1 or

AXIN2 mutations. No mutations were detected in any of the normal tissues. This result indicated that AXIN1 and AXIN2 mutations may be solely responsible for the disruption of the Wnt signaling pathway in GC cases.

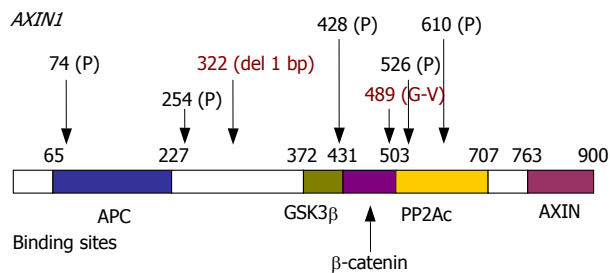


Figure 2 Mutations and polymorphisms found in AXIN1. Bold lines indicate the coding regions corresponding to each protein-binding site. GSK3 β : Glycogen synthase kinase 3 β ; PP2Ac: Protein phosphatase 2Ac; P: Polymorphism.

The functional importance of AXIN1, AXIN2 and CTNNB1 mutations in GC development was further analyzed by immunohistochemical analysis of β -catenin in 50 available GC tissue samples. Ten (20%) of the GCs showed increased nuclear immunoreactivity for β -catenin compared with non-cancerous tissues. All four GCs with AXIN1 and AXIN2 mutations showed nuclear β -catenin expression.

DISCUSSION

Activation of Wnt signaling is an important step in the development of human tumors^[23,24]. APC is part of a multiprotein complex in the Wnt pathway which induces β -catenin degradation^[25]. AXIN1, AXIN2, GSK-3 β , β -catenin, PP2A and PP2C are additional components of this complex^[26]. In this complex, AXIN1 and AXIN2 play major roles as scaffold proteins. Mutations in CTNNB1 or APC have been reported in GC^[17,18]. To explore if AXIN1 and AXIN2 are also involved in the pathogenesis of GC, we examined mutations in the entire coding region of AXIN1, exon7 of AXIN2 and exon3 of CTNNB1 in 70 GCs. Four (5.7%) of the 70 GCs had mutations in AXIN1, one had frameshift mutations in AXIN2 and one had a mutation in CTNNB1. Our results showed that AXIN1 and AXIN2 are likely to be tumor suppressor genes involved in the carcinogenesis of GC. This is the first study, to our knowledge, to demonstrate AXIN1 and AXIN2 mutations in GC.

AXIN1 mutations have been reported in hepatocellular carcinomas, hepatoblastomas and sporadic medulloblastomas^[10,11]. We have now identified genetic alterations of AXIN1 in GCs. We found a 1-bp deletion at nucleotide 1076, which has previously been reported in hepatocellular carcinomas, in three samples^[11]. This mutation interrupts the GSK3 β and β -catenin binding domain. Additionally, we discovered a somatic point mutation in GCs, resulting in a substitution of the amino acid at 489 from glycine to valine, a site located within the β -catenin binding domain. It is possible that such alterations may alter protein secondary structure and thereby the interaction of β -catenin with APC/GSK-3 β complex. Since AXIN1 is a negative regulator of β -catenin, failure to form a complex with any one of these key players of Wnt signaling is likely to stabilize β -catenin and lead to its accumulation.

AXIN2 mutations were found in one of the 70 GCs. Such mutations resulted in the elimination of the DIX

Table 2 The profile of AXIN1 polymorphisms

Exon	Nucleotide changes	Amino acid ¹	Frequency (%)
1	334 C→T	Gly74Gly	4.3
	874 C→T	Asp254Asp	42.8
5	1396 G→A	Ser428Ser	10.0
	1690 C→T	Asp526Asp	2.9
6	1942 T→G	Ala610Ala	12.9

¹Amino acid number begins from Met at position 113 of GenBank accession, No. AF009674.

domain, which is necessary for AXIN oligomerization^[27-29]. This case had both AXIN1 and AXIN2 mutations, and showed increased nuclear immunoreactivity for β -catenin. AXIN2 mutations have been previously reported in colorectal cancers (in 10 of 11 cases) and were also accompanied by increased nuclear β -catenin staining^[7], similar to our results in GC. This study also showed that frameshift mutations in AXIN2 appear to be specifically associated with defective DNA mismatch repair (MMR) in colorectal cancer^[7]. To determine the MSI status, we identified two mononucleotide markers, BAT26 and BAT25, by DHPLC^[30]. This case was detected as MSI-H. Our results indicate that GCs with MMR deficiency often harbor somatic frameshift mutations in cancer-related genes.

CTNNB1 mutations were identified in 70 GCs. A 27-bp deletion spanning exon3 of CTNNB1 was observed in one of the cases, which showed increased membrane and cytoplasmic immunoreactivity for β -catenin. We did not detect any missense mutations of this gene in our GC cases. CTNNB1 mutations occur even more frequently in colon cancer and hepatoblastoma^[11,12]. A number of studies have identified CTNNB1 mutations in GCs, but the frequencies of these mutations were significantly different in those studies^[17,31]. The different frequencies of CTNNB1 mutations identified could be due to differences in the populations examined or the method of microdissection of tumor cells used. Analysis of β -catenin in a large sample set using a precise method of microdissection is needed to delineate the functional importance of this gene in GC development.

In conclusion, our results indicate that AXIN1 and AXIN2 mutations may contribute to the pathogenesis of GC *via* activation of the Wnt signaling pathway. Further studies will be required to investigate the function of mutated AXIN1 and AXIN2 variants reported in this study.

COMMENTS

Background

The Wnt signaling pathway is known to be involved in tumorigenesis. Recently, AXIN1 and AXIN2, components of Wnt signaling pathway, were characterized as new candidate tumor suppressor genes that may be targeted for deletion or mutation during tumorigenesis.

Research frontiers

Mutations in AXIN1, AXIN2 and CTNNB1 were identified by PCR-based denaturing high-performance liquid chromatography (DHPLC) and direct DNA sequencing. Beta-catenin expression was detected by immunohistochemical staining.

Innovations and breakthroughs

A total of 5 (7.1%) GCs had mutations in one or two of these three components. A frameshift mutation in exon7 of AXIN2 was found in one case. Four cases had frameshift mutations and missense mutations in AXIN1, and 5 single nucleotide polymorphisms (SNPs) were identified in AXIN1. All four cases with mutations in AXIN1 and AXIN2 showed nuclear beta-catenin expression.

Applications

AXIN1 and AXIN2, key players in the Wnt signaling pathway, are involved in gastric carcinogenesis, but the functions of mutated AXIN1 and AXIN2 variants need to be further investigated.

Peer review

This is an interesting report of mutations in components of the Wnt signaling pathway in 70 patients with gastric cancer. They examined AXIN1, AXIN2 and CTNNB1 and found mutations in only 5 (7.1%) of the patients. This is an important and well-written paper.

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Angiopoietin-1 targeted RNA interference suppresses angiogenesis and tumor growth of esophageal cancer

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Abstract

AIM: To determine the inhibitory effect of the adenovirus-based angiopoietin-1 (Ang-1) targeted small interfering RNA expression system (Ad/Ang-1si) on the expression of the Ang-1 gene, cell growth and apoptosis in human esophageal cancer cell line Eca109.

METHODS: siRNA-expressing adenovirus targeting Ang-1 gene was constructed using the Ad Easy System. Cultured Eca109 cells were transfected with Ad/Ang-1si (Eca109/Ang-1si), and Ad/si was used to infect Eca109 cells as control (Eca109/si). Ang-1 gene expression and concentration was determined with RT-PCR and ELISA, respectively. Human umbilical vein endothelial cell (HUVEC) migration and proliferation were analyzed. After s.c. injection into athymic nu/nu mice, the tumor growth, vessel density and apoptosis of each group was also determined.

RESULTS: HUVEC migration induced by conditioned medium from Ang-1si-transfected Eca109 cells was significantly less than that induced by conditioned medium from Eca109 cells and control adenovirus-transfected Eca109 cells. Furthermore, after s.c. injection into athymic nu/nu mice, the tumor growth and cell apoptosis of Ad/Ang-1si-expressing Eca109 cells was significantly lower than that of parental or control adenovirus-transfected cells. Vessel density assessed by CD31 immunohistochemical analysis and Ang-1 expression by RT-PCR were also decreased.

CONCLUSION: The targeting Ang-1 may provide a therapeutic option for esophageal cancer.

INTRODUCTION

Human esophageal cancer is the eighth most frequently diagnosed cancer and the sixth most frequent cause of cancer death in the world^[1]. Esophageal cancer is one of the most lethal cancers of the digestive tract with a very low survival of 16% in the United States and 10% in Europe^[2]. Most esophageal cancers arise from the middle and lower third of the esophagus. Despite the use of multimodal therapy (chemotherapy, radiation therapy, and surgery), the long-term disease-free survival rate of patients with esophageal cancer is still disappointingly low, particularly in the high-risk groups^[3]. The identification of new therapeutic targets is therefore needed.

Angiogenesis has been specifically linked to increased growth and metastatic potential in human tumors^[4-9]. Although numerous growth factors are involved, angiopoietin-1 (Ang-1) play a pivotal role in tumor angiogenesis^[10-17]. Binding of Ang-1 to its ligand of the Tie-2 receptor reduces endothelial permeability and enhances vascular stabilization and maturation. All these events which contribute to angiogenesis and remodeling of blood vessels. However, Ang-1 contribution to tumor growth has received little attention.

Esophageal cancer is vascular in nature, with a high proliferation rate^[18]. Several lines of evidence indicate a role of Ang-1 in the pathogenesis of esophageal cancer. Loges *et al* recently reported 94% of tumor specimens expressed Ang-1, implying a close association between Ang-1 expression and microvessel density (MVD)^[19]. High levels of Ang-1 expression were also detected by immunohistochemical analysis in 42 of 45 esophageal cancer samples, and positive staining for Ang-1 at the time of diagnosis

was correlated with VEGF121 and VEGF165 gene expression^[20]. Taken together, these data indicate that Ang-1 is associated with neovascularization in the cancer stroma through VEGF networks in esophageal cancer, and inhibition of the expression or function of Ang-1 may improve in the disease outcome.

Our data in this study showed that adenoviruses-based siRNA expression system can be used to down-regulate Ang-1 expression, resulting in suppression of cell growth and induction of apoptosis through inhibiting angiogenesis in a nude mouse model of esophageal cancer. It can be concluded that Ang-1 is an alternative target in developing new therapeutic strategies for the treatment of esophageal cancers.

MATERIALS AND METHODS

siRNA-expressing adenovirus targeting angiopoietin-1

siRNA-expressing adenovirus targeting human Ang-1 was constructed according to the manufacturer's instructions. Briefly, one pairs of oligonucleotides targeting human Ang-1 mRNA was synthesized by Sagon DNA technologies (Sagon Biotechnology). The targeted Ang-1 sequences were 5'-GATCCCGAGGCTG-GAAGGAATATAATTCAAGAGATTATATTCCTTC-CAGCCTCTTTT-3', 5'-AGCTAAAAAAGAGGCTG-GAAGGAATATAATCTCTTGAATTATATTCCTTC-CAGCCTCGG-3'. The dsDNA was ligated between the BamHI and HindIII sites on the pShuttle containing H1 promoter and GFP sequences. Adenoviruses were then constructed using the Ad Easy System (Stratagene Biotechnology). The control vector was constructed by inserting a sequence that expresses a siRNA with limited homology to sequences in the human and mouse genomes. Adenoviral DNA was prepared on a large scale in *Escherichia coli* DH5 α , generating Ad/Ang-1si and Ad/si, respectively. All adenoviruses were propagated in HEK293 cells and purified using BD Adeno-XTM purification kit (BD Biosciences Clontech). Viral titers were determined using BD Adeno XTM rapid titer kit (BD Biosciences Clontech).

Cell culture and transfection

Eca109 human esophageal cancer cell line was obtained from Shanghai Institute of Cell Biology, Chinese Academy of Sciences. Eca109 cells were cultured in DMEM with 10% fetal bovine serum. Eca109 cells were infected with the Ad/Ang-1si at 50 PFU/cell (Eca109/Ang-1si), Ad/si was used to infect Eca109 cells as control (Eca109/si). Adenovirus generation was confirmed by the expression of GFP. The ECV2304 endothelial cell line, derived from immortalized human umbilical vein endothelial cells (HUVEC) was purchased from Shanghai Institute of Cell Biology and grown in DMEM containing 10% FCS, 2 mmol/L glutamine, HAT (hypoxanthine 0.1 mmol/L, aminopterin 0.4 mmol/L, thymidine 16 mmol/L), and antibiotics. HUVECs (passage 3 or 4) with ~80% confluent were used for most experiments.

Cell growth assay

Eca109, Eca109/si and Eca109/Ang-1si cells were har-

vested and reseeded at 1×10^4 cells/well in 12-well plates. The total cell number was determined every two days with a hemacytometer and under an inverted microscope (Olympus). Cell viability was detected by trypan blue staining. Each value represents the average of triplicate wells.

RT-PCR for angiopoietin-1

Total RNA was isolated from Eca109, Eca109/si and Eca109/Ang-1si cells using the Trizol protocol (Invitrogen Biotechnology). Reverse transcription reactions were carried out for 1 h at 42°C with 1 μ g of total RNA, 250 ng of oligo(dT), $1 \times$ deoxynucleotide triphosphate mix, RNase inhibitor (Promega), $1 \times$ RT buffer, and 200 units of SuperScript II RT (Invitrogen) in a total volume of 20 μ L. Amplification of Ang-1 was performed in 50 μ L of reaction mixture consisting of sense and antisense primers for Ang-1: 2 μ g of cDNA, $1 \times$ deoxynucleotide triphosphate mix, $1 \times$ PCR buffer, 1.5 mmol/L MgCl₂, and 2.5 units of AmpliTaq Gold DNA Polymerase (Perkin-Elmer, Wellesley, MA). The following primers were used: 5'-ATGACAG TTTTCCTTTCC-3', 5'-TCAAAAATCTAAAGGTCG-3' (Sagon Biotechnology).

Quantification of secreted angiopoietin-1 protein

Eca109, Eca109/si and Eca109/Ang-1si cells (2.5×10^5) were seeded into 24-well plates. Fresh medium was added after overnight culture. The cultured supernatants were collected 24 h later and centrifuged to eliminate cellular fragments. Ang-1 protein accumulated in the culture medium was analyzed using sandwich ELISA, wherein the supernatant of the culture was incubated with Ang-1 antibody (goat polyclonal anti-human Ang-1, Santa Cruz Biotechnology) and streptavidin alkaline phosphatase (Santa Cruz Biotechnology). The antigen-antibody complex was then incubated with p-nitrophenyl phosphate (Sigma Biotechnology) dissolved in pNPP buffer (Chemicon Biotechnology). Ang-1 concentrations in the samples were determined from the absorbance at 570 nm spectrophotometrically.

Cell migration assay

Cultured supernatants from Eca109, Eca109/si, and Eca109/Ang-1si cells were collected. Transwells (Costar, Cambridge, MA) were pretreated with serum-free medium at 37°C for 1 h before seeding with HUVECs at 1×10^5 per well in 100 μ L endothelial basal medium with 0.1% fetal bovine serum. The transwells were then inserted into 24-well plates containing 600 μ L conditioned medium and incubated at 37°C for 6 h to allow HUVEC cells to migrate. Cells on the upper side of the filter were removed with cotton swabs. Migrated cells on the lower side of the filter were fixed and stained with HE. The number of migrated cells was counted under a binocular microscope.

Cell proliferative assay

HUVEC cells (2.5×10^5) were seeded into 96-well plates, and allowed to adhere for 5 h. The metabolic activity of HUVEC cells was determined every two days by methyl thiazoleterazolium (MTT) assay. Briefly, after light rinsing with PBS, 40 μ L 5 g/L MTT (Sigma Biotechnology) was

added. After 4 h of incubation at 37°C, HUVEC cells were washed with PBS, and then dissolved in 150 μ L dimethyl sulfoxide (DMSO). The absorbance of each group was determined spectrophotometrically ($\lambda = 570$ nm).

Tumorigenicity assays

Four- to 5-wk-old specific pathogen-free athymic (T-cell deficient) nude mice were purchased from Shanghai Experimental Animal Supply, China. The animal protocol was approved by the Institutional Animal Care and Utilization Committee of the Second Military Medical University. Eca109, Eca109/si, and Eca109/Ang-1si cells in mid-log-growth phase were harvested by trypsinization. Single-cell suspensions (2×10^6 cells in 0.1 mL HBSS) were injected s.c. into the nude mice. The tumors were measured every 4 d with a caliper, and the diameters were recorded. Tumor volume was calculated by the formula: $a^2b/2$, where a and b are the two maximum diameters. When tumors reached $2 \text{ cm} \times 2 \text{ cm}$, the mouse was euthanized, and the tumor tissue was collected, fixed in formalin, embedded in paraffin, cut into 3 μ m sections, and stained with HE.

Microvessel counting

Tissue sections stained with CD31 was used for evaluating MVD. Immunohistochemical staining was performed using the two step immunohistochemical technique with the DAKO EnVision system (EnVisionTM + Kits, HRP, DAKO Biotechnology). For antigen retrieval, the slides were treated with boiling 0.01 mol/L citrate buffer (pH 6.0) for 15 min. Tissue sections were incubated at room temperature for 1 h with CD31 antibody (goat polyclonal anti-human CD31, Santa Cruz Biotechnology), followed by incubation with horseradish peroxidase-labeled goat anti-mouse immunoglobulin for 30 min and developed with 3, 3'-diaminobenzidine. Then the slides were examined under $\times 100$ magnification for the hot spots rich in vessels and MVDs were counted under $\times 200$ magnification ($0.708 \text{ mm}^2/\text{field}$), so that every single brown-stained cell and cell cluster was calculated as a blood vessel, no matter whether a vessel lumen structure was seen. For vessels with large lumens, every 40 μ m length of lumen was calculated as one vessel. Five different fields were chosen on each of the slides, and the stained vessels were counted simultaneously by 2 researchers under a multiocular microscope. The average of the 5 areas was recorded as the MVD score.

In situ apoptosis detection by TUNEL assay

The TUNEL assay was performed for detection of *in situ* apoptotic cell death according to the manufacturer's instructions (*in situ* cell death detection kit, HRP; Roche Biotechnology). Briefly, the sections were treated with proteinase K (20 mg/L) for 30 min and incubated at room temperature for 1 h with TUNEL reaction mixture, 50 μ L peroxidase conjugate was added to each slide and the slides incubated for 30 min. For color reaction, 3, 3'-diaminobenzidine was used. The apoptotic index was expressed as the number of TUNEL-positive nuclei per 1000 cells counted for each sample.

Statistical analysis

The data were expressed as mean \pm SE. Statistical compar-

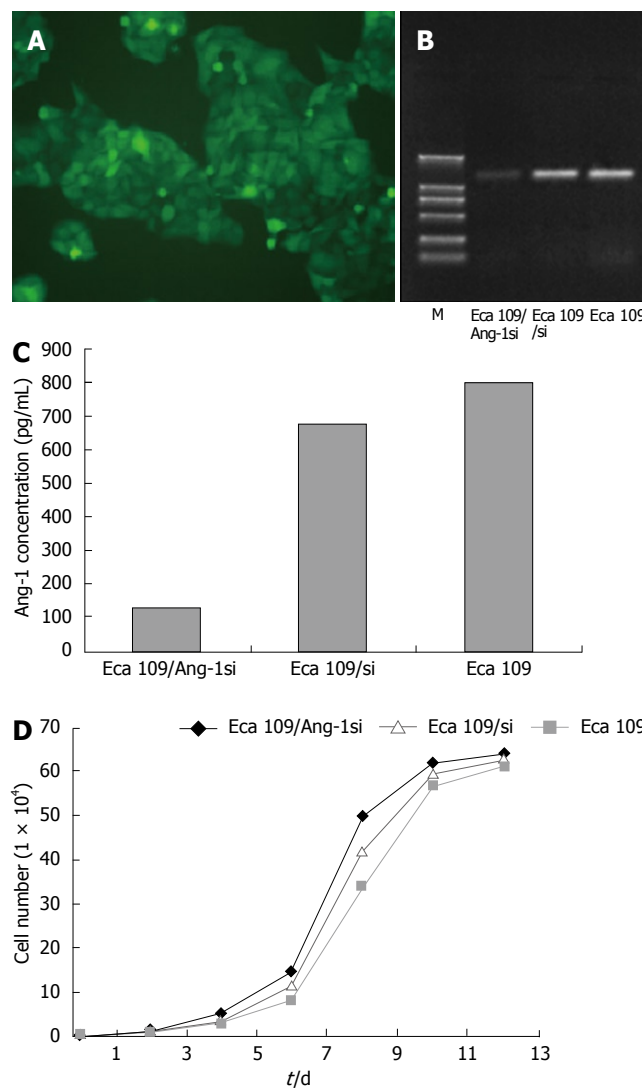


Figure 1 Effect of Ang-1si on Ang-1 expression in Eca109 esophageal cancer cells. **A:** Eca109 cells were infected with Ad/Ang-1si at a multiplicity of infection (MOI) of 50, nearly 100% of cultured Eca109/Ang-1si cells were GFP-positive under the fluorescent microscope; **B:** Ang-1 mRNA was quantified by RT-PCR. Compared with Eca109, the Ang-1 mRNA level of Eca109/Ang-1si cells was reduced by 80%, but no significant alteration in Eca109/si cells; **C:** Secreted Ang-1 protein was quantified by ELISA. Compared with Eca109, the Ang-1 concentration in the media was decreased by Ad/Ang-1si, but no significant change in the cell line transfected with Ad/si; **D:** Cell number was measured at various time points, there was no difference of cell growth curve among Eca109, Eca109/si, and Eca109/Ang-1si cells.

ison was made using SPSS10.0 statistical software package. The criterion for statistical significance was $P < 0.05$.

RESULTS

Effect of angiopoietin-1 small interfering RNA on angiopoietin-1 expression

Adenovirus expressing siRNA to Ang-1 was constructed using the Ad Easy System to target human Ang-1 mRNA. Eca109 cells were stably transfected with this adenovirus, the transfection efficiency being approximately 100% (Figure 1A). The Ang-1 mRNA level was measured using RT-PCR, as shown in Figure 1B, Ang-1 expression was significantly inhibited by Ang-1si in Eca109/Ang-1si cells and Ang-1 level was reduced by 80% compared with Eca109

and Eca109/si cells. ELISA was performed using human Ang-1-specific antibody to quantify the amount of Ang-1 protein in the culture media. When cells were transfected with adenovirus expressing siRNA to Ang-1, the Ang-1 concentration in the media was decreased significantly (Figure 1C) compared with Eca109 and Eca109/si cells ($P < 0.05$). These results were in agreement with the amount of Ang-1 mRNA analyzed by RT-PCR experiments.

In addition, cell number was measured at various time points and no difference of cell growth curve was found among Eca109, Eca109/si, and Eca109/Ang-1si cells (Figure 1D).

Effect of angiopoietin-1 small interfering RNA on angiopoietin-1 induced endothelial cell proliferation and migration

We collected cultured supernatants from Eca109, Eca109/si, and Eca109/Ang-1si cells. As shown in Figure 2A, the culture supernatants from Eca109 and Eca109/si cells induced robust endothelial cells migration. By contrast, few endothelial cells migrated when conditioned medium from Eca109/Ang-1si cells was used as the chemotactic stimulus.

To determine whether Ang-1 inhibition by siRNA influenced endothelial cells proliferation, the proliferative activity of each cell line was quantified by MTT. However, endothelial cells proliferation was not significantly lower when cells were cultured for 48 h in conditioned medium from Eca109/Ang-1si cells rather than from untransfected or control adenovirus-transfected cells (Figure 2B).

Effect of angiopoietin-1 small interfering RNA on esophageal cancer growth in vivo

To determine whether inhibition of Ang-1 by siRNA had an effect on tumor growth, Eca109, Eca109/si, or Eca109/Ang-1si cells was inoculated s.c. into *nu/nu* mice. Eca109 and Eca109/si cells grew rapidly, resulting in palpable tumors 3-4 d following injection. By contrast, tumor formation was significantly slow after inoculation of Eca109/Ang-1si. The Eca109/Ang-1si tumors were significantly smaller than those in both control groups (Figure 3A).

H&E staining showed that Eca109/Ang-1si tumors were pale, with a massively necrotic center and a thin layer of tumor cells in the periphery (Figure 3B). On the contrary, control tumors appeared very vascularized.

As shown in Figure 3C, CD31-positive vessels were abundant in Eca109 and Eca109/si tumors. MVD was significantly decreased in tumors formed by Eca109/Ang-1si, although numerous vessels were seen in the normal tissues surrounding the tumor (Figure 3D). The MVD in tumors treated with Eca109/si was similar to that observed in the Eca109 tumors.

Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling assay revealed the presence of massive apoptotic and necrotic cells in mice inoculated with Eca109/Ang-1si tumors. Eca109 and Eca109/si tumors showed only small areas of necrosis and apoptosis. Figure 3D shows that the apoptotic cells significantly increased in Eca109/Ang-1si tumors compared to Eca109 and Eca109/si tumors (Figure 3, $P < 0.05$).

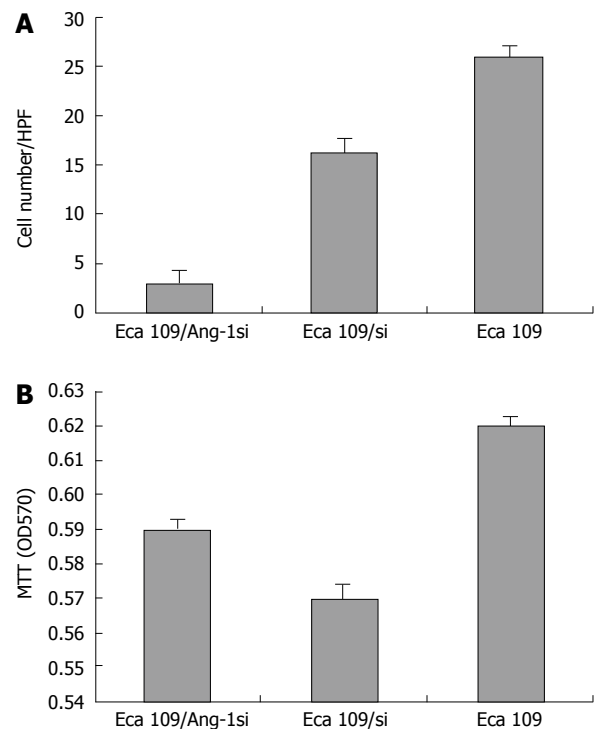


Figure 2 Effect of Ang-1si on HUVEC cell proliferation and chemotaxis. **A:** HUVEC cell migration was quantified after 6 h, few HUVEC cells migrated as the chemotactic stimulus from Eca109/Ang-1si cells supernatants compared with Eca109 and Eca109/si cells supernatants. $^bP < 0.01$; **B:** HUVEC cells were incubated with the indicated supernatant for 48 h, no difference of proliferative activity of Eca109/Ang-1si cells from Eca109 and Eca109/si cells. $^bP < 0.01$.

DISCUSSION

Several studies have shown that Ang-1 is abundantly expressed in the primary tumors of esophageal cancer patients. In the present study, we showed that Ang-1 played a critical role in the growth of esophageal cancer. This was done by selectively inhibiting Ang-1 expression and protein production using RNA-mediated interference by siRNA.

RNA interference is a phenomenon that can be used to block specific gene expression, and has advantages over other strategies because of its high selectivity and potency. Delivery of small interfering RNA (siRNA) can be achieved through exogenous application of synthetic siRNA or through endogenous expression using plasmid or vector delivery to the target cells^[21,22]. Chemically or enzymatically synthesized siRNA is costly and has been shown to have a relatively short half-life with only transient inhibition of the target gene because of its low transfection rate and short duration of interference. Moreover, DNA vector-based siRNA technology has some advantages over chemically or enzymatically synthesized siRNA, as it induces more efficient and stable RNA interference^[23,24]. However, DNA plasmid vector induced siRNA still has limitations with respect to the clinical application of siRNA technology due to its low transfection efficiency.

To overcome these shortcomings, we constructed an adenovirus-based expression system in which sense and antisense strands of short Ang-1 sequence was transcribed into hairpin structures under the control of a H1 promoter and then processed into functional siRNAs by double

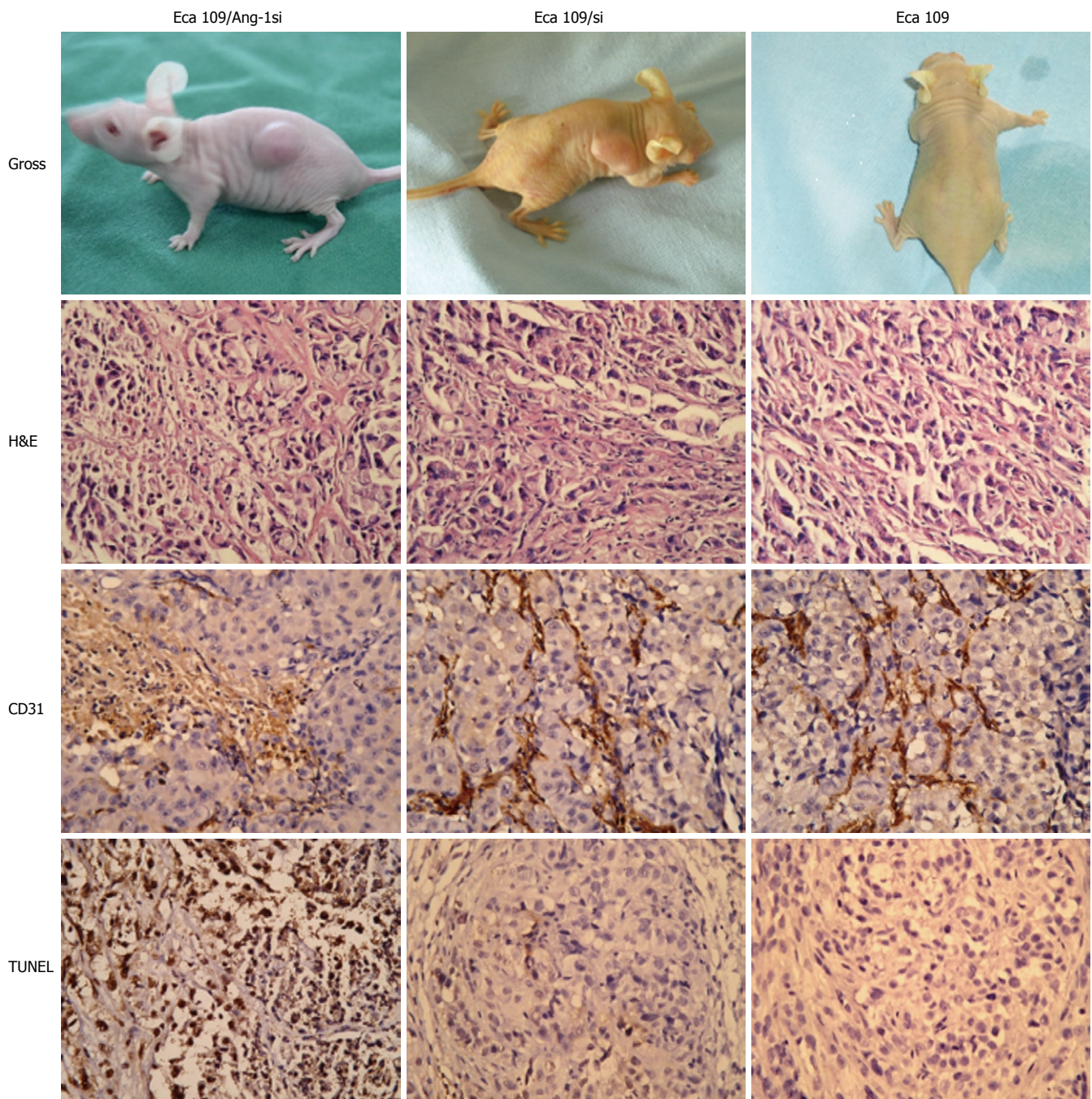


Figure 3 Effect of Ang-1si on esophageal cancer growth *in vivo*. Grossly, the Eca109/Ang-1si tumors were significantly smaller than those in both control groups; Under microscopy, massive necrotic tissue was found in Eca109/Ang-1si tumors, while Eca109 and Eca109/si tumors showed fine necrosis; the Eca109/Ang-1si tumors were avascular with lower MVD, while Eca109 and Eca109/si tumors appeared very vascularized with higher MVD; Eca109/Ang-1si tumors underwent massive apoptosis with more TUNEL-positive cells compared with Eca109 and Eca109/si tumors, which showed a finely granular cytoplasm with evenly dispersed chromatin and lower TUNEL-positive cells.

strand-specific RNase. In this study, we used the highly selective siRNA approach to adenoviral constructs for genetic blockade of Ang-1 with a transfection efficiency of approximately 100%. Ang-1si specifically blocked Ang-1 expression in Eca109 human esophageal cancer cell line. Reduction in Ang-1 protein production was also documented by the finding that HUVEC cell migration induced by Eca109 conditioned medium was almost completely abolished following transfection with Ad/Ang-1si but it was not affected by the Ad/si control adenovirus. By contrast, HUVEC cell proliferation was unchanged in both

Eca109/Ang-1si and Eca109/si cells. These results were in agreement with the reports of Koblizek *et al* that Ang-1 was not an endothelial cell mitogen^[25,26]; however, it stimulated endothelial cell migration^[27-30].

Transfection of Ad/Ang-1si into Eca109 cells did not alter the cell growth *in vitro*. However, when these cells were injected either s.c. or into the bone of nude mice, tumor growth was slower than in parental and Ad/si control-transfected cells. Eca109/Ang-1si cells produced small tumors that were avascular in appearance with decreased vessel density and increased apoptotic and necrotic cells.

Together, these data confirmed once again the specificity of our Ang-1si and indicated that Ang-1 played a pivotal role in esophageal cancer angiogenesis and tumor growth through promoting tumor neovascularization independently from promotion of tumor cell proliferation.

In summary, we have applied the highly selective siRNA approach to adenoviral constructs for genetic blockage of Ang-1 with a high transfection rate and long duration of interference. We found that siRNA technology can be used to specifically inhibit Ang-1 expression. *In vitro* promotion of angiogenesis in Ang-1 protein was documented by the finding that HUVEC cell migration induced by Eca109 conditioned medium was almost completely abolished following transfection with Ad/Ang-1si. *In vivo* cell transfection of Ad/Ang-1si resulted in selective inhibition of Ang-1 expression, leading to decreased tumor vascularity and growth *in vivo*. These data indicated that Ang-1 played a central role in esophageal cancer angiogenesis. Therefore, targeting Ang-1 with specific small-molecule inhibitors may have therapeutic benefit.

COMMENTS

Background

Angiopoietin-1 (Ang-1) has been shown to play a pivotal role in tumor angiogenesis. Several lines of evidence indicate that Ang-1 is associated with neovascularization in esophageal cancer, and inhibition of the expression or function of Ang-1 may improve the disease outcome. This study showed that adenovirus-based small interfering RNA (siRNA) expression system can be used to down-regulate Ang-1 expression, resulting in suppression of cell growth and induction of apoptosis through inhibiting angiogenesis in a nude mouse model of esophageal cancer.

Research frontiers

Angiogenesis has been found to be specifically linked to increased growth and metastatic potential in human tumors. Study in this field has become one of the hot spots at present. Previous studies demonstrated that growth factors, especially Ang-1, are responsible for the growth of esophageal cancer. Based on these findings, treatment targeting this gene has been designed and studied in *in vivo* and *in vitro* models. Preliminary results of these studies have shown beneficial and promising effects. Further experimental and clinical studies are needed before certain conclusions can be reached.

Innovations and breakthroughs

The association between angiopoietin-1 and angiogenesis and human cancer has been studied previously. However, the inhibitory effect of the adenovirus-based Ang-1 targeted small interfering RNA expression system (Ad/Ang-1si) on the expression of the Ang-1 gene, cell growth and apoptosis in Eca109 has not been explored. This study has bridged this gap and may provide additional targets for therapeutic development.

Applications

Since some basic evidence provided for angiopoietin-1, angiogenesis and their relationships in the development of human cancer, therapeutic approaches targeting angiopoietin-1 can be implemented in future studies and development of new methods for human cancers.

Terminology

Angiopoietin-1 (Ang-1): Angiopoietin, a group of proteins including four molecules with similar structure, belongs to a growth factor family. Ang-1 is an endothelium-specific growth factor that can promote angiogenesis. As a 70 kDa glycoprotein, it is mainly produced by juxtavascular cells including pericytes, vascular smooth muscle cells and tumor cells, affecting the surrounding vascular endothelial cells through paracrine action. It can promote migration of endothelial cells, anti-apoptosis, and formation of luminal structure. Thus, it plays an important role in angiogenesis of human cancer.

Peer review

This is an informative study demonstrating that Ang-1 played a central role in esophageal cancer angiogenesis. Therefore, targeting Ang-1 with specific small-molecule inhibitors may have therapeutic benefit. The preliminary conclusion is justified and substantiated by the results obtained.

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RAPID COMMUNICATION

Refined mapping of loss of heterozygosity on 1q31.1-32.1 in sporadic colorectal carcinoma

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the critical and precise deleted region was located within 2 cM chromosomal segment encompassing 2 loci (D1S413, D1S2622). No significant association was found between LOH and clinicopathologic features in 1q31.1-32.1.

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Key words: Sporadic colorectal carcinoma; Loss of heterozygosity; Tumor suppressor genes; 1q31.1-32.1

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Zhou CZ, Qiu GQ, Fan JW, Wang XL, Tang HM, Huang L, Sun YH, Peng ZH. Refined mapping of loss of heterozygosity on 1q31.1-32.1 in sporadic colorectal carcinoma. *World J Gastroenterol* 2008; 14(10): 1582-1587 Available from: URL: <http://www.wjgnet.com/1007-9327/14/1582.asp> DOI: <http://dx.doi.org/10.3748/wjg.14.1582>

Abstract

AIM: To explore precise deleted regions and screen the candidate tumor suppressor genes related to sporadic colorectal carcinoma.

METHODS: Six markers on 1q31.1-32.1 were chosen. These polymorphic microsatellite markers in 83 colorectal cancer patients tumor and normal DNA were analyzed *via* PCR. PCR products were electrophoresed on an ABI 377 DNA sequencer. Genescan 3.1 and Genotype 2.1 software were used for Loss of heterozygosity (LOH) scanning and analysis. Comparison between LOH frequency and clinicopathological factors was performed by χ^2 test.

RESULTS: 1q31.1-32.1 exhibited higher LOH frequency in colorectal carcinoma. The average LOH frequency of 1q31.1-32.1 was 23.0%, with the highest frequency of 36.7% (18/49) at D1S2622, and the lowest of 16.4% (11/67) at D1S412, respectively. A minimal region of frequent deletion was located within a 2 cM genomic segment at D1S413-D1S2622 (1q31.3-32.1). There was no significant association between LOH of each marker on 1q31.1-32.1 and the clinicopathological data (patient sex, age, tumor size, growth pattern or Dukes stage), which indicated that on 1q31.1-32.1, LOH was a common phenomenon in all kinds of sporadic colorectal carcinoma.

CONCLUSION: Through our refined deletion mapping,

INTRODUCTION

The progression of the colorectal carcinoma is thought to result from an accumulation of genetic alteration at numerous loci controlling growth and proliferation^[1-3]. As a model for both multistep and multipathway carcinogenesis, colorectal neoplastic progression provides paradigms of both oncogenes and tumor suppressor genes^[1,2]. The loss of heterozygosity (LOH), the loss of one paternal or maternal allele at specific locus, on tumor suppressor genes is believed to be one of the key steps to colorectal carcinogenesis^[3,4]. When this occurs at a tumor suppressor gene locus where one of the alleles is already abnormal, it can result in neoplastic transformation^[5]. According to the previous study, in colorectal carcinomas, frequent allelic loss was identified in chromosome 5q (30%)^[3,6], 8p (40%)^[3,7], 17p (75%-80%)^[3,8], 18q (80%)^[3,9], and 22q (20%-30%)^[3,10,11]. Moreover tumor suppressor genes APC, p53, and DCC were found, which were located on chromosome 5q, 17p, and 18q, respectively^[12-14]. The LOH analysis on sporadic carcinoma by means of microsatellite markers has become an effective way to find allelic deletion regions and then to find candidate

tumor suppressor genes^[3,15,16]. In a previous study we used microsatellite markers to analyze the LOH at 21 loci on chromosome 1 in sporadic colorectal carcinoma. We found that D1S468 (1p36.33-36.31, 9.4 cM) and D1S413 (1q31.1-32.1, 9.8 cM) exhibited higher LOH frequencies, which indicated that the two regions might harbor putative tumor suppressor gene(s)^[17,18]. However, the allelic deletion region found in our previous studies contained about 50 genes, which was inconvenient for further gene screening and functional studies. Therefore, further LOH scanning with high-density microsatellite markers in the two regions was necessary to narrow the research scope and select fewer candidate genes in the finite regions to functional research. In this study, other six markers from 1q31.1-32.1, at a density of approximately one marker every 1.67 cM, were chosen to analyze refined LOH mapping of the region.

MATERIALS AND METHODS

Subjects

This study was based on 83 cases of sporadic colorectal carcinoma, comprising 40 males and 43 females, treated at the surgical department in Shanghai Jiaotong University Affiliated First People's Hospital, China, between 1998 and 1999. Ages ranged from 31 to 84 years with a median of 66 years. All patients were confirmed by pathology and were staged by Dukes criterion. Their distribution according to their clinical stages was as follows: Dukes stage A, 8 cases; stage B, 21 cases; stage C, 40 cases; stage D, 14 cases. Their distribution, according to cancer location, was as follows: proximal colon cancer, thirty-three cases; distal colon, 21 cases; and rectal, 29 cases. The carcinomas were confirmed, *via* pathological examination, as being well differentiated adenocarcinoma in 23 patient cases, moderate differentiated adenocarcinoma in 39 cases, poorly differentiated adenocarcinoma in 6 cases, mucinous adenocarcinoma in 15 cases. HNPCC patients were ruled out by the Amsterdam criteria^[19,20]. Each patient gave his or her informed consent for the use of his or her tissue in this study.

DNA extraction

The cancerous and adjacent normal tissues were frozen within 30 min after removal. The tissues were then cut into cubes of approximately 2 mm³ and immediately frozen in liquid nitrogen. DNA was extracted by standard methods with proteinase K digestion and phenol/chloroform purification^[21].

Microsatellite markers and PCR

By searching in Genothon, NCBI and GDB databases, 6 polymorphic microsatellite markers were chosen, at a density of approximately one marker every 1.67 cM, covering the chromosomal region 1q31.1-32.1 and spanned the D1S413 locus. Based on the databases described previously, the order of these markers was centromere-D1S2877-D1S412-D1S2757-D1S413-D1S2622-D1S2683-D1S2668-qter. The primer sequences are shown in Table 1. Polymorphic microsatellite markers were analyzed in each patients' tumor and normal DNAs by PCR

Table 1 The primer sequences of seven microsatellite markers

Primer	3'-5'	5'-3'
D1S2877	AGACATTNCATTGAAGTCTAT TTAT	CAAGCCACTAGCGTA AGAGC
D1S412	TAGGACTTTTCAAC TTCCACAG	ATAGGCACAGAATC AATGAATG
D1S2757	TTTTTAATGACTGACCAAGTG	TGCCTTCGTCTATGTTTG
D1S413	GCCAAGCCTGAGATCAAAAT	ACTTGAACAGATTGGGATTG
D1S2622	CTGCAACATAAGAACCTAG TGTAAC	AAACTGGTAGGCCATT GATAGA
D1S2683	TGCCCTGTCTTCAAGAGC	GCAGTGACAGGAATCTGG
D1S2668	AATCACTGAACCTGGGAG	ACTGACTGGCTGTTCTGAG

(GeneAmp PCR System 9700, PE Applied Biosystems Foster city CA, USA). PCR conditions were as follows: 5 μ L total volume with 1 μ L (1.4 ng) DNA as a template with 10 \times standard buffer, 0.3 μ L MgCl₂, 0.8 μ L deoxynucleotide triphosphates, 0.3 unit of Hot-start Taq polymerase and 0.06 μ L of each primer, with the forward primer fluorescence labeled with FAM (Shanghai Shengggong Biological Engineering & Technology and Service Co. Ltd, China), and fill ddH₂O up to 5 μ L. Cycling conditions consisted of 3 stages: an initial denaturation at 96°C for 12 min in Stage I; 14 cycles each at 94°C for 20 s, 63-56°C for 1 min (0.5°C decreased per cycle), 72°C for 1 min, in Stage II; 35 cycles each at 94°C for 20 s, 56°C for 1 min, 72°C for 1 min in stage III^[17,18, 22-24].

LOH analysis

A portion of each PCR product (0.5 μ L) was combined with 0.1 μ L of Genescan 500 size standard (PE Applied Biosystems Foster city CA, USA) and 0.9 μ L of formamide loading buffer. After denaturation at 96°C for 5 min, products were electrophoresed on a 5% polyacrylamide gels on an ABI 377 DNA sequencer (PE Applied Biosystems Foster city CA, USA) for 3 h. Genotype 2.1 software displayed individual gel lanes as electrophoretograms with a given size, height, and area for each detected fluorescent peak. Stringent criteria were used to score the samples. Alleles were defined as the two highest peaks within the expected size range. A ratio of T1:T2/N1:N2 of less than 0.67 or greater than 1.50 was scored as a LOH (Figure 1). Most amplification of normal DNA produced two PCR products indicating heterozygosity. A single fragment amplified from normal DNA (homozygote) and PCR reactions in which fragments were not clearly amplified were scored as not informative. The LOH frequency of a locus was equal to the ratio of the number between allelic loss and informative cases. The average LOH frequency was the average value of each locus LOH frequency^[17,18, 22-24].

Statistical analysis

Comparison between LOH and clinicopathological data was performed by χ^2 test. $P < 0.05$ was considered statistically significant.

RESULTS

LOH analysis on 1q31.1-32.1

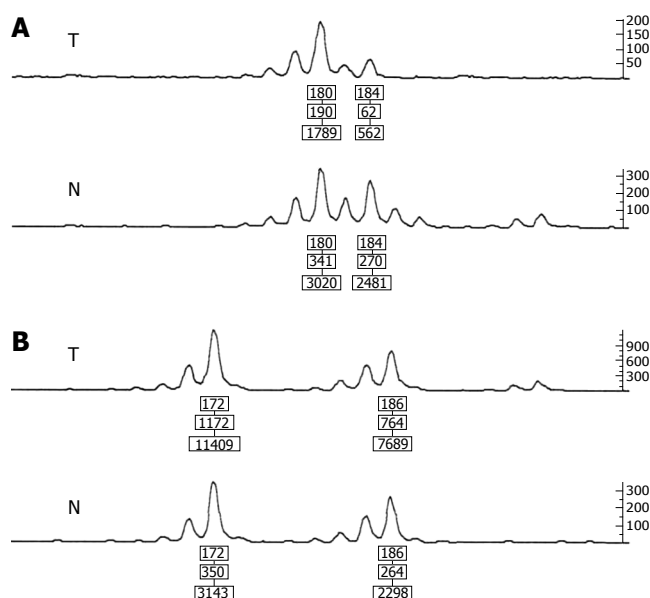


Figure 1 The LOH and normal peak. **A:** The typical peak of LOH: Allele ratio = (T1/T2)/(N1/N2) = (190/62)/(341/270) = 2.43 > 1.5; **B:** The peak of normal (no LOH): Allele ratio = (T1/T2)/(N1/N2) = (1172/764)/(350/264) = 1.15; T: Tumor; N: Normal.

The average LOH frequency of 1q31.1-32.1 was 23.0%, with the highest frequency of 36.7% (18/49) at D1S2622, and the lowest of 16.4% (11/67) at D1S412, respectively. According to this study, a minimal region of frequent deletion was located within a 2 cM genomic segment at D1S413-D1S2622 on 1q31.3-32.1 (Table 2).

Relationship between clinicopathological features and LOH frequency

There was no significant association between LOH of each marker on 1q31.1-32.1 and the clinicopathological data (patient sex, age, tumor size, growth pattern or Dukes stage). It indicated that on 1q31.1-32.1, LOH was a common phenomenon in all kinds of sporadic colorectal carcinoma (Table 3).

DISCUSSION

During tumorigenesis, loss of the wild-type allele is frequently observed at the appropriate locus. It has been admitted that LOH on tumor suppressor genes plays a key role in colorectal carcinoma transformation. LOH is common to all human solid tumors and allows expressivity of recessive loss of function mutations of tumor suppressor genes. Therefore, detection of recurrent LOH in a chromosome region is now considered critical evidence of localization of tumor suppressor genes^[3,25-28]. In the previous study, initial LOH scanning was carried out in 83 sporadic colorectal carcinoma samples with 21 highly polymorphic markers on chromosome 1. We found that D1S468 (1p36.33-36.31) and D1S413 (1q31.1-32.1) exhibited higher LOH frequencies^[17,18]. However, the average genetic distance of the loci in our previous study was 9.6 cM, which contained so many genes that was inconvenient for further gene screening. Further LOH scanning with high-density microsatellite markers in the

Table 2 LOH frequencies of microsatellite loci on 1q31.1-32.1 in colorectal cancer

Locus	Location	LOH case	Normal case	LOH rate (%)	Informative rate (%)
D1S2877	1q31.1	7	29	19.44	43.37
D1S412	1q31.3	11	56	16.42	80.72
D1S2757	1q31.3	18	43	29.51	73.49
D1S413	1q31.3	13	27	32.50	48.19
D1S2622	1q32.1	18	31	36.73	59.04
D1S2683	1q32.1	10	41	19.61	61.45
D1S2668	1q32.1	4	20	16.67	28.92

two regions was necessary to narrow the research scope and select fewer candidate genes in the finite regions to functional research. In another study of ours, we carried out the refined LOH mapping on 1p36.33-36.31 and found two critical and precise deleted regions, D1S243 (1 cM) and D1S468-D1S2660 (3 cM)^[29]. In this study, six high-density polymorphic microsatellite markers were chosen for refined LOH mapping of LOH on 1q31.1-32.1 in order to get much more genetic information and to screen the potential tumor suppressor genes.

The results showed that the average LOH frequency of 1q31.1-32.1 was 23.0%, with the highest frequency of 36.7% (18/49) at D1S2622, and the lowest of 16.4% (11/67) at D1S412, respectively. According to this study, a minimal region of frequent deletion was located within a 2 cM genomic segment at D1S413-D1S2622 on 1q31.3-32.1. There are few reports in past years about the relationship between the long arm of chromosome 1 and colorectal carcinoma. Moreover, some previous studies showed that 1q frequently presents allelic loss in other tumors, such as breast cancer, medulloblastoma, thyroid cancer, sporadic insulinoma and esophageal carcinoma. Benitez's^[30] study showed more than 60% of the breast tumors exhibited allelic loss in the 1q31-32 region. Pietsch *et al*^[31] found that 36% of the medulloblastomas showed LOH on 1q31-32.1. Moreover accordingly to the study of Kitamura^[32], frequent allelic loss was identified on 1q31-42 (40%) in anaplastic thyroid carcinomas. Yang *et al*^[33] found that thirty-five out of forty (88%) insulinomas had 1q LOH of the 35 insulinomas with 1q LOH, 14 (40%) had 1q21.3-23.2 LOH over a 7.5 cM region, whereas in 21 tumors (60%) LOH occurred at 1q31.3 over an 11.4 cM area. Li *et al*^[34] analyzed LOH in 61 esophageal squamous cell carcinomas using 18 microsatellite markers on chromosome 1q. Forty-six of 61 tumors (75.4%) presented LOH at one or more loci. These results suggested that putative tumor suppressor genes might locate on the 1q. Our study firstly demonstrated that 1q31-32 exhibited higher LOH frequency in colorectal carcinoma, which suggesting the presence of a tumor suppressor gene in this region. This gene might be involved in the tumorigenesis of colorectal carcinoma and other tumors. Based on our study, allelic deletion was located within 2 cM chromosomal segment encompassing 2 loci (D1S413, D1S2622). Searching the databases, no known tumor suppressor genes have been found in this region. However, we presumed CSRP1 might

Table 3 The relationship between clinicopathological features and LOH of the loci on 1q31.1-32.1

		D1S2877		D1S412		D1S2757		D1S413		D1S2622		D1S2683		D1S2668	
		T ¹	N ²	T	N	T	N	T	N	T	N	T	N	T	N
Gender	Male	4	13	3	27	10	18	7	14	11	15	5	20	2	5
	Female	3	16	8	29	8	25	6	13	7	16	5	21	2	15
Age(yr)	> 60	5	24	9	39	13	32	10	18	16	22	10	29	2	16
	≤ 60	2	5	2	17	5	11	3	9	2	9	0	12	2	4
Location	Proximal colon	2	10	6	22	5	18	6	10	9	13	6	13	0	9
	Distal colon	2	8	4	15	5	11	3	7	5	8	0	14	1	7
	Rectum	3	11	1	19	8	14	4	10	4	10	4	14	3	4
Gross pattern	Massive	2	15	5	25	7	22	5	14	6	16	3	22	1	9
	Ulcerative	4	13	5	23	9	18	5	9	9	14	6	17	1	7
	Encroaching	1	1	1	8	2	3	3	4	3	1	1	2	2	4
Size (cm)	≥ 5	4	12	7	22	6	18	6	11	8	15	5	17	2	9
	< 5	3	17	4	34	12	25	7	16	10	16	5	24	2	11
LN metastasis	(+)	4	20	6	39	12	29	6	16	15	20	5	26	1	16
	(-)	3	9	5	17	6	14	7	11	3	11	5	15	3	4
Differentiation	Well	3	4	1	14	5	7	3	7	4	5	1	10	3	5
	Moderately	2	21	8	26	8	27	6	12	10	18	5	25	0	12
	Poorly	1	1	2	2	2	2	1	2	1	2	2	1	0	1
	Mucinous	1	3	0	14	3	7	3	6	3	6	2	5	1	2
Dukes stage	A	2	5	2	3	3	3	2	3	1	3	2	5	0	1
	B	1	4	3	14	3	11	5	8	2	8	3	10	3	3
	C	3	16	4	29	11	19	4	12	10	17	3	19	0	13
	D	1	4	2	10	1	10	2	4	5	3	2	7	1	3

T: Tumor; N: Normal.

be the candidate colorectal carcinoma related gene in this region. At the mRNA level, the highest concentrations of CSRP1 were found in the prostate and the colon followed by the brain and the testis^[35]. CSRP1 was a member of the CSRP family of genes encoding a group of LIM domain proteins, which might be involved in regulatory processes important for development and cellular differentiation^[36]. We supposed that the inactivation of this genes could lead to the abnormality of cell differentiation and then result in neoplastic transformation. Recently, Hirasawa *et al.*^[37] found that CSRP1 were inactivated in HCC by aberrant methylation and they may serve as important biomarkers of malignancy, indicating that CSRP1 was a the tumor related gene. Further functional research will provide additional proof. In order to find the colorectal carcinoma related gene in 1q31.1-32.1, we are performing a microarray-based high-throughput gene screening approach in this region to identify unknown candidate related genes, and this may provide much more genetic information and find the potential tumor suppressor gene(s). In our study, there was no significant association between LOH of all loci on 1q31.1-32.1 and the clinicopathological data, indicating that in this region, LOH was a common phenomenon in all kinds of sporadic colorectal cancer.

In summary, through our detailed deletion mapping studies, the critical and precise deleted region was located within 2 cM chromosomal segment encompassing 2 loci (D1S413, D1S2622). No significant association was found between LOH and clinicopathologic features. The region identified in the present study might harbor one or more tumor suppressor genes, which might be involved in several types of human cancer. Our study provided the significant data to reveal the mechanism of

colorectal carcinogenesis. And further microarray-based high-throughput gene screening and functional research may provide much more genetic information and find the potential tumor suppressor genes.

COMMENTS

Background

The loss of heterozygosity (LOH) analysis on sporadic carcinoma by means of microsatellite markers has become an effective way to find allelic deletion regions and then to find candidate tumor suppressor genes. In a previous study Zhou *et al* found that D1S413 (1q31.1-32.1, 9.8 cM) exhibited higher LOH frequencies and this indicated that this region might harbor the putative tumor-suppressor-gene(s). However, the allelic deletion region the previous studies found contained so many genes that it was inconvenient to further gene screening and functional study. Therefore, in this study, another six markers with high-density microsatellite markers in 1q31.1-32.1 were chosen to analyze refined LOH mapping to narrow the research scope and select fewer candidate genes in the finite regions to functional research.

Research frontiers

LOH on tumor suppressor genes is believed to be one of the key steps to colorectal carcinogenesis. According to the previous study, in colorectal carcinomas, frequent allelic loss was identified in chromosome 5q (30%), 8p (40%), 17p (75%-80%), 18q (80%), and 22q (20%-30%). Subsequently, tumor suppressor genes APC, p53, and DCC were found, which were located on chromosome 5q, 17p, and 18q, respectively. The LOH analysis on sporadic carcinoma by means of microsatellite markers has become an effective way to find allelic deletion regions and then to find candidate tumor suppressor genes.

Innovations and breakthroughs

This study found that the precise deleted region was located within 2 cM chromosomal segment encompassing 2 loci (D1S413, D1S2622), in which might harbor one or more tumor suppressor genes related to the colorectal carcinogenesis.

Terminology

LOH: The loss of one allele at a specific locus, caused by a deletion mutation; or loss of a chromosome from a chromosome pair, resulting in abnormal hemizyosity. It is detected when heterozygous markers for a locus appear monomorphic because one of the alleles was deleted. When this occurs at a tumor suppressor gene locus where one of the alleles is already abnormal, it can result in neoplastic transformation.

Peer review

It is possible that 1q will be an informative site in colorectal carcinogenesis. But, it may also simply reflect the chromosomal instability (CIN) pathway of genomic instability that was selected for in the selection of predominantly distal, non-mucinous cancers. CIN causes wide spread aneuploidy, and thus LOH in the 1q31.1-32.1 region may be an epiphenomenon, rather than a sentinel event in carcinogenesis. Nevertheless, this study provides some important information about this region. It would be interesting to know whether they have looked at gene expression of CSRP1 in any of their samples, or in any CRC cell lines, to support their assertion that this is a likely candidate in this region.

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RAPID COMMUNICATION

Colorectal carcinoma-associated antigen Ca-Hb3 detected by one-dimensional SDS-polyacrylamide gel electrophoresis and liquid chromatography-tandem mass spectrometry

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Abstract

AIM: To comprehensively identify the proteins of tumor relative antigen Ca-Hb3 recognized by colorectal carcinoma monoclonal antibody Hb3.

METHODS: Ca-Hb3 was isolated by SDS-polyacrylamide gel electrophoresis (PAGE) followed by digestion with trypsin. Trypsin peptides were analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS). The proteins identified by mass spectrometry were analyzed using bioinformatics.

RESULTS: Ca-Hb3 was identified as a CKAP4-like protein by Nano HPLC tandem mass spectrometry analysis. The molecular weight of CKAP4-like protein was 62.02 kDa, including one hydrophobic region, one transmembrane domain, five coiled coils, four glycosylation sites and forty-nine phosphorylation sites. CKAP4-like protein had a high homogeneity with DeltaNp63 α . The characteristic expression of DeltaNp63 α that is considered a potential oncogene in the isoforms of p63 was similar to that of Ca-Hb3.

CONCLUSION: Ca-Hb3 is probably a CKAP4-like protein, belonging to DeltaNp63 α isoform of p63 family.

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Key words: Colorectal neoplasm; Monoclonal antibody; Antigen; Mass spectrometry; p63

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INTRODUCTION

Colorectal cancer can be considered a complex disease with predisposing genetic variants and environmental factors that contribute to the illness as a whole. In recent years, many studies reported that the incidence of colorectal cancer is increased by 4% per year in China^[1-3]. Hb3 is an anti-colorectal cancer monoclonal antibody produced in our laboratory. Its sensitivity and specificity are superior to those of anti-CEA. Monoclonal antibody Hb3 (mAb Hb3) targets the Hb3 antigen which is expressed in 85% of colorectal cancers, showing that CA-Hb3 may be useful in the diagnosis of colorectal cancer. In this study, using SDS-polyacrylamide gel electrophoresis (PAGE) and microcapillary reversed-phase liquid chromatography-tandem mass spectrometry, we have identified 5 proteins, including one membrane protein which was initially determined to be Ca-Hb3 by bioinformatics analysis.

MATERIALS AND METHODS

Preparation of mAb Hb3

mAb Hb3, a murine IgM-type mAb, was prepared using 5×10^8 Hb3 hybridoma cells (our laboratory) to immunize Balb/c mice. Mice were sacrificed and ascites was extracted to obtain supernatant by centrifugation.

Instrumentation

NanoLC-MS was performed online with a Waters capillary LC system interfaced with a Waters (Micromass) electrospray ionization quadrupole time-of-flight (ESI-Q-TOF) mass spectrometer.

Cells and cell culture

Human colon carcinoma HRT-18 cells were cultured

in RPMI 1640 medium (Promega Inc.) supplemented with 100 mL/L heat-inactivated fetal bovine serum (FBS, Hangzhou Sijiqing BRL Inc.), 30 g/L *L*-glutamine, 100 kg/L streptomycin and 100 U/L penicillin at 37°C in a humidified atmosphere containing 50 mL/L CO₂.

SDS-PAGE and Western blot analysis

Total cell extraction from human colon carcinoma HRT-18 cells was performed by Western blot assay. Samples in the cold lysate buffer (50 mmol/L Tris-Cl pH 8.0, 150 mmol/L NaCl, 10 mmol/L Triton X-100, 10 mmol/L PMSF) were electrophoresed on 10% gradient SDS-polyacrylamide gels in the presence of β -mercaptoethanol. Half of the proteins were transblotted onto a NC membrane, which was blocked with 50 mg/mL fat-free milk and 100 mL/TBS for 2 h and incubated with primary antibody (mAb Hb3) overnight at 4°C. The membrane was washed and incubated with HRP-conjugated goat anti-mouse IgG/IgM (SIGMA Inc.) at room temperature for 1 h. The membrane was washed and the antigen-antibody reaction was visualized using an ECL detection system (KPL Inc.). The other half of proteins were stained with Coomassie blue.

Trypsin digestion

Bands of interest were excised and minced into 1 mm³ pieces with sterile razor blade. The pieces were washed 3 times with ddH₂O and then with 50% acetonitrile in 100 mmol/L NH₄HCO₃, incubated in water bath for 60 min if necessary. The liquid was discarded, 100 μ L 1% acetonitrile was added and the dried acetonitrile was removed after 10 min. For SDS-PAGE gel bands, gel pieces were rehydrated and proteins were reduced with 200 μ L of 100 mmol/L DTT in 100 mmol/L ammonium bicarbonate for 30 min at 56°C. The reduction solution was discarded and 100 μ L of 50 mmol/L iodoacetamide in 100 mmol/L ammonium bicarbonate was added. Incubation lasted for 30 min at room temperature in the dark. Gel bits were washed with 500 μ L of 25 mmol/L ammonium bicarbonate in 500 mL/L acetonitrile for 15 min, then 100 μ L 100% acetonitrile was added and the dried acetonitrile was removed after 10 min. The gel pieces were rehydrated by adding 5 μ L of trypsin in 40 mmol/L ammonium bicarbonate and incubated overnight at 37°C. The supernatant was removed and placed into a new tube. Peptides were extracted from the gel pieces with 50 μ L of 20 mmol/L ammonium bicarbonate for 20 min and then with 50 μ L of 5% formic acid in 50% acetonitrile for 2 \times 20 min. The extracts were added to the supernatant. The mixed solution was dried using a Speed-Vas.

Nano-HPLC tandem mass spectrometric analysis

RP-HPLC separation of the peptide samples was performed using a bioinert ultimate nano-HPLC system (Dionex, Sunnyvale, CA, USA). Six microliters of each sample was injected and peptides were purified and concentrated on a C18-PepMap precolumn (0.3 mm ID \times 65 mm, 100Å pore size, 3 mm particle size, Dionex) at a flow rate of 0.02 mL/min. Subsequently, peptides were separated on an analytical 75 mm ID \times 150 mm C18-PepMap column (Dionex, 100Å pore size, 3 mm particle

size) at a column flow rate of 300 mL/min. The ACN gradient (solution A: 1 mL/L formic acid, 20 mL/L ACN; solution B: 1 mL/L formic acid, 800 mL/L ACN) started at 50 mL/L B and ended at 70 mL/L B in 45 min. MS and MS/MS data were collected using a Micromass Q-TOF micro mass spectrometer (Waters, Milford, MA, USA). Doubly and triply charged peptide ions were automatically chosen by the MassLynx software (Waters) and fragmented for a maximum of 7 s for each component. MS data were automatically processed and peak lists for database search were generated by the MassLynx software. Database search was carried out with an in-house MASCOT server using an IPI protein database (Mouse, version 3.07), for a mass tolerance of 5 nkat and allowance for up to 16.67 nKat trypsin miscleavage and variable amino acid modifications consisting of methionine oxidation and cysteine carbamidomethylation.

Data analysis and bioinformatics

Tandem mass spectrometry (MS/MS) data were collected by the Mass-Lynx 4.0 software (Waters), deisotoped and converted by Waters ProteinLynx Global server 1.0 software to "peak list" (pkl) files, which include the mass values, the intensity (at least 5 counts/s) and the charge of precursor ions. The parameters used in the creation of pkl files were the following background subtraction below curve 10%, which was smoothed three times with "smooth window" (channels) 2.0 in the Savitzky Golay mode, centroid at 80% top and minimum peak width at half height at 4. The pkl files were analyzed using a licensed copy of the Mascot2.0 Program (MatrixScience Ltd., London) on a 2.6 GHz Pentium-4 personal computer with 2 GB of RAM, which compared the files against a human protein non-redundant database containing 146724 protein sequences downloaded as FASTA formatted sequences from NCBI. Search parameters were set as follows: enzyme, trypsin, allowance of up to one missed cleavage peptide, mass tolerance, 0.3u MS/MS mass tolerance, fixed modification parameter, carbamoylmethylation (C), variable modification parameter, oxidation (at Met), auto hits allowed (only significant hit report), result format as peptide summary report. Predictions for putative transmembrane domains (TMDs) in all identified proteins were carried out using the transmembrane hidden Markov model (TMHMM) algorithm, available at <http://www.cbs.dtu.dk/services/TMHMM>.

RESULTS

Ca-Hb3 was characterized by Western blot analysis (Figure 1) and one specific electrophoretic band of about 65 kDa in the cell lysates was shown, supporting the previous experiment results^[3]. Ca-Hb3 crude antigen was separated by one-dimensional SDS-PAGE (Figure 2). Proteins present in the interest band were digested by trypsin. The tryptic peptide was extracted from the purpose band and further separated by reversed-phase nanoLC, detected and sequenced with a Waters Q-TOF micro mass spectrometer. Raw data were analyzed by Mascot ms/ms ion search against the human nonredundant database.



Figure 1 Western blot analysis of Ca-Hb3 crude antigen.

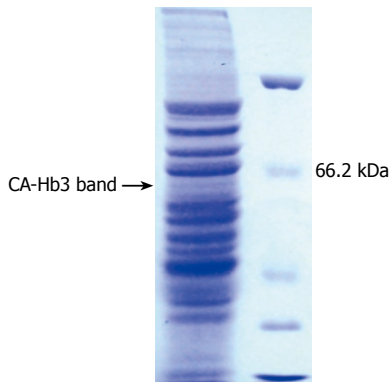


Figure 2 One-dimensional SDS-PAGE image of Ca-Hb3 crude antigen.

Using the stringent criteria mentioned above, we identified 5 non-redundant proteins: one inactivate hybrid protein, three nuclear proteins and one CKAP4-like protein. The molecular weight of the second protein (Chain A, crystal structure of the Tpr2a domain of Hop in complex with the Hsp90 peptide Meevd) showed 15484u by MS, and was thus judged to be the inactivate hybrid protein. We found that only CKAP4-like protein (similar to cytoskeleton-associated protein 4) belonged to an integral membrane protein (<http://www.expasy.org>), consistent with the previous experiment results (Figure 3).

DISCUSSION

Hb3 is an IgM monoclonal antibody against CA-Hb3^[1-3]. The results of immunohistochemistry showed that the positive rate of Hb3 reacting with colorectal carcinoma was high.

To characterize the proteins of complex proteomes, the proteins were analyzed by 2D-PAGE followed by MS or MS/MS, or by 2D-LC-MS/MS. Because Ca-Hb3 is located in membrane, it is difficult to resolve membrane proteins by 2D-PAGE. We replaced the 2D-PAGE and 2D-LC with a one-dimensional SDS-PAGE. By applying the method mentioned above, we identified 5 non-redundant proteins. Except for an inactivate hybrid protein and three nuclear proteins, the remaining CKAP4-like protein had a transmembrane domain at the corresponding Ca-Hb3 location.

The p63 gene, independently cloned by multiple laboratories, is a member of the p53 family of transcription factors^[4-6]. p53 is a tumor suppressor that is inactivated in a majority of human cancers. In response to cellular stresses, such as DNA damage and oncogene activation, p53 is stabilized and acts as a sequence-specific transcription factor, trans-activating target genes are involved in the processes of cell cycle arrest, DNA repair, and apoptosis^[7-10]. These proteins exhibit a high sequence and structural

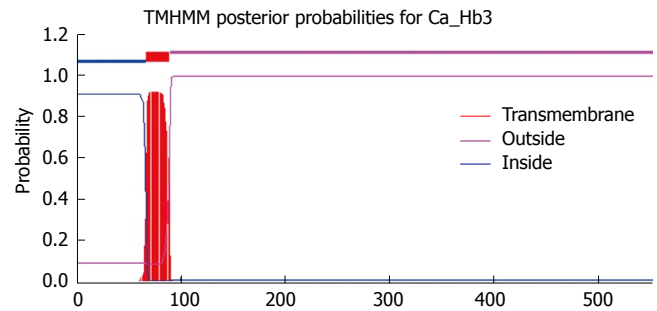


Figure 3 Relationship between cellular membrane and CKAP4-like protein.

similarity to p53, while revealing a considerable functional divergence^[11-15].

The human p63 gene consisting of 15 exons, resides on chromosome 3q27-29 and can be expressed in two different promoters^[16,17]. Transcription from the first and second promoters gives rise to TA or ΔN amino terminus of p63. Both TA and ΔN transcripts can be alternatively split at the carboxy-terminus, leading to formation of α, β and γ isoforms of TA and ΔN p63α. A number of studies have highlighted the oncogenic potential of ΔN p63α which is over-expressed in several epithelial cancers, often as a result of gene amplification^[18-23]. Over-expression of ΔN p63α isoform in rat-1A cells increases colony growth in soft agar and xenograft tumor formation in nude mice, supporting that p63 acts as an oncogene^[19]. Furthermore, ΔNp63α can prevent p53-mediated trans-activation, growth arrest, and apoptosis^[24]. The expression of ΔNp63α is associated with epithelial homeostasis, poor survival of ovarian cancer patients, and promotes keratinocyte adhesion, inhibits apoptosis, and maintains the integrity of epidermal tissue^[25,26]. ΔNp63α regulates CD44 and keratins 4, 6, 14 and 19 in squamous cell carcinoma of the head and neck, and down-regulation of DeltaNp63α acquires invasive phenotype of human squamous cell carcinoma^[27,28]. It is hypothesized that ΔNp63α prolongs survival and maintains the proliferating capacity of epithelial stem and cancer cells.

In conclusion, colon cancer monoclonal antibody Hb3 is probably a CKAP4-like protein, belonging to DeltaNp63α isoform of p63 family, which can be considered a marker for colon cancer progression or as a therapeutic target.

COMMENTS

Background

Hb3 is an anti-colorectal cancer monoclonal antibody produced in our laboratory. Its sensitivity and specificity are superior to those of anti-carcinoembryonic antigen (anti-CEA), showing that CA-Hb3 might be useful in the diagnosis of colorectal cancer.

Research frontiers

There are a number of researchers who are searching for a useful marker of tumor. Antigen, as an effect target, is applied in clinical therapy for colon cancer.

Innovations and breakthroughs

ΔNp63α obtained by one-dimensional polyacrylamide gel electrophoresis (PAGE) and liquid chromatography-tandem mass spectrometry could be considered a marker for colon cancer progression or as a therapeutic target.

Applications

Δ Np63 α could be applied in treatment of cancer patients as an effect target.

Terminology

DeltaNp63 α is an isoform of P63 family. Δ Np63 α is over-expressed in several epithelial cancers, often as a result of gene amplification. Over-expression of a Δ Np63 α isoform in rat-1A cells increases colony growth in soft agar and xenograft tumor formation in nude mice.

Peer review

This paper is well organized with valuable conclusions.

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RAPID COMMUNICATION

Inhibition of hepatitis B virus replication by pokeweed antiviral protein *in vitro*

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Abstract

AIM: To explore the inhibitory effects of pokeweed antiviral protein seed (PAP-S) and PAP encoded by a eukaryotic expression plasmid on hepatitis B virus (HBV) replication *in vitro*.

METHODS: HepG2 2.2.15 cells in cultured medium were treated with different concentrations of PAP-S. HBsAg, HBeAg and HBV DNA in supernatants were determined by ELISA and fluorescent quantitative PCR respectively. MTT method was used to assay for cytotoxicity. HepG2 were cotransfected with various amounts of PAP encoded by a eukaryotic expression plasmid and replication competent wild-type HBV 1.3 fold over-length plasmid. On d 3 after transfection, HBsAg and HBeAg were determined by using ELISA. Levels of HBV core-associated DNA and RNA were detected by using Southern and Northern blot, respectively.

RESULTS: The inhibitory effects of PAP-S on HBsAg, HBeAg and HBV DNA were gradually enhanced with the increase of PAP concentration. When the concentration of PAP-S was 10 µg/mL, the inhibition rates of HBsAg, HBeAg and HBV DNA were 20.9%, 30.2% and 50%, respectively. After transfection of 1.0 µg and 2.0 µg plasmid pXF3H-PAP, the levels of HBV nucleocapside-associated DNA were reduced by 38.0% and 74.0% respectively, the levels of HBsAg in the media by 76.8% and 99.7% respectively, and the levels of HBeAg by 72.7% and 99.3% respectively as compared with controls. Transfection with 2 µg plasmid pXF3H-PAP reduced the levels of HBV nucleocapside-associated RNA by 69.0%.

CONCLUSION: Both PAP-S and PAP encoded by a eukaryotic expression plasmid could effectively inhibit HBV replication and antigen expression *in vitro*, and the inhibitory effects were dose-dependent.

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Key words: Pokeweed antiviral protein; Hepatitis B virus; Antiviral agent

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INTRODUCTION

Pokeweed antiviral protein (PAP), a 29-kDa protein isolated from leaves or seeds of *Phytolacca americana*, inhibits translation by catalytically removing a specific adenine residue from the large rRNA of the 60S subunit of eukaryotic ribosomes^[1,2]. The anti-viral activity of PAP has been described against a wide range of viruses, including HIV, herpes simplex virus, cytomegalovirus, influenza virus and polio virus^[3,4]. Dose response studies indicate that the anti-HIV activity of PAP from PAP-S was comparable to Zidovudine (AZT)^[5].

The purpose of the present study was to evaluate “*in vitro*” the potential usefulness of PAP in therapies against chronic hepatitis B. Hepatitis B virus (HBV) is a major cause of liver disease worldwide, ranging from acute and chronic hepatitis to cirrhosis and hepatocellular carcinoma^[6]. Despite the availability of an effective and safe vaccine against HBV, infection by this virus is an important worldwide health problem^[7]. Although several pharmacological strategies are currently being implemented to treat affected patients, no effective antiviral therapy

against HBV infection has yet been fully developed^[8]. Thus, new drugs to be used alone or in combination with existing treatments are needed. In this respect, “*in vitro*” screening of potentially active compounds is a useful step in the development of novel drugs.

In this study, we assessed anti-HBV activities of PAP from PAP-S by detecting HBsAg, HBeAg, and HBV DNA in the supernatant of HepG2 2.2.15 cell lines. We also constructed the eukaryotic expression plasmid encoding PAP, cotransfected it and the replication competent wild-type HBV 1.3 fold over-length plasmid into the HepG2 cells to investigate the inhibitory effect of the eukaryotic expression plasmid of PAP. PAP was found to be a remarkably potent and fast-acting antiviral agent against HBV replication *in vitro* in the absence of any obvious signs of toxicity. This is the first report of the anti-HBV effects of PAP. Our observations suggest that PAP deserves further investigation as a potential alternative or complementary anti-HBV agent.

MATERIALS AND METHODS

Plasmid constructs and reagents

The plasmid PGEM-PAP containing the PAP gene was kindly supplied by Prof. YAN Bo (Institute of Biotechnology, Yunnan Academy of Agricultural Sciences, China). The replication competent wild-type HBV 1.3 fold over-length plasmid (genotype, ayw) and the plasmid pXF3H derived from the cytomegalovirus-driven vector pRK5 were both kindly supplied by Dr. Lei Yanchang. The coding gene of PAP (Gene Bank GI: 218010) was amplified from the plasmid PGEM-PAP by PCR using forward primer 5'-CGGGATCCAATAAATACAATCACCTTC GTAA-3' and reverse primer 5'-CC AAGCTTAGGGAA CATGGCACTTTGGTAA-3'. The PCR product was cloned into *Bam*H I / *Hind*III restriction sites of the CMV-driven expression vector fused with a hemagglutinin fusion epitope tag at its N-terminus (pXF3H).

PAP-S, purified from pokeweed (*Phytolacca americana*) seeds, was kindly supplied by Professor HU Zhong (Kunming Institute of Botany, The Chinese Academy of Sciences, China).

Cell culture and treatment

HepG2 and HepG2 2.2.15 cells were grown at 37°C with 50 mL/L CO₂ in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum (FCS) (Invitrogen, CA, USA).

HepG2 cells were seeded on plastic dishes (3.5 cm in diameter) at a density of 4.5×10^5 cells per well in 6-well plates 18 h prior to transfection. Transfection of HepG2 cells was performed with lipofectamine 2000 (Invitrogen, CA, USA) following the manufacturer's instructions. The replication competent wild-type HBV 1.3 fold over-length plasmid pHBV1.3 and various amounts of a CMV-driven expression vector pXF3H-PAP were cotransfected into the HepG2 cells.

HepG2 2.2.15 cells were treated with PAP-S at various concentrations for 2 or 4 d in DMEM supplemented with 10% FCS in a 24-well plate at a density of 2×10^4 cells

per well. The corresponding suspension was collected for analysis of the levels of HBsAg, HBeAg, and HBV DNA, in duplicate every 2 d for 4 d. The HepG2 2.2.15 cells as controls were washed twice with phosphate-buffered saline (PBS) and refed with culture medium every 2 d for 4 d.

HBsAg and HBeAg assays and Western blot analysis

The concentration of HBsAg or HBeAg in culture supernatants of HepG2 2.2.15 or the transfected HepG2 cells was detected by an enzyme-linked immunosorbent assay (ELISA) kit (Shanghai Kehua Company, China) and quantified relative to a standard curve of serial dilutions of recombinant HBsAg or HBeAg. Inhibition rate (%) = (P/N values of the control wells - P/N values of the test wells) / (P/N values of the control wells - 2.1) \times 100%, and the median inhibition dose (ID₅₀) represented the concentration of PAP-S when the inhibition rate was 50%. The median cytotoxin dose (CD₅₀) represented the concentration of PAP-S when the number of the survival cells was 50%.

In order to determine the efficiency of transfection, Western blot with polyclonal anti-HA antibody was used to detect the expression of PAP in cotransfected HepG2 cells. For Western blot analysis, cytoplasmic lysates were incubated with 1 volume of $2 \times$ loading buffer containing 10% beta-mercaptoethanol for 10 min at 95°C before loading onto a 12.5% SDS-PAGE. Proteins were transferred onto a nitrocellulose membrane via electroblot. The membranes were incubated with anti-hemagglutinin fusion epitope polyclonal antibody (Santa Cruz, USA) followed by horseradish peroxidase conjugated mouse anti-rabbit antibody. Proteins were visualized via enhanced chemiluminescence (Roche, Germany).

Supernatant HBV DNA from HepG2 2.2.15 cells analysis by quantitative real-time PCR

For HBV DNA from HepG2 2.2.15 cells, the DNA was detected by real-time PCR kit (DA AN GENE CO., Guangzhou, China) following the instruction manual provided. Amplification and detection were performed with an ICycler (Bio-Rad) Detection system. The program was optimized with denaturation at 94°C for 2 min followed by 40 cycles of amplification (at 94°C for 20 s, 55°C for 20 s, and 72°C for 20 s).

Detection of HBV core-associated DNA and mRNA by Southern and Northern blot in cotransfected HepG2 cells

On d 3 after transfection, HepG2 cells were lysed with 0.8 mL of 0.01 mol/L Tris-HCl (pH 8.0), 0.05 mol/L NaCl, 5 mL/L NP-40, 1 mmol/L EDTA at room temperature for 10 min as previously described^[9]. For the Southern hybridizations, 20 µg of total DNA was digested with *Hind*III, electrophoresed on a 1.4% agarose gel, and then transferred onto nylon membrane. The probe containing the full-length HBV genome was labeled with digoxigenin dUTP (DIG) by the DIG-high Prime DNA labeling and Detection Starter kit II according to the manufacturer's protocol (Roche, Germany). The membrane was hybridized with HBV probe at 50°C overnight.

For Northern blot analysis, total RNA was extracted from the transfected cells with TRIZOL reagent (Gibco, USA) according to the manufacturers instructions. A total of 20 µg of RNA was resolved in 1.2% denatured gel and then transferred onto the nylon membrane and the membrane was hybridized with DIG-labeled HBV DNA fragment described above. For hybridization of glyceraldehyde-3-phosphate dehydrogenase (G3PDH), the full-length HBV DNA probe was removed from the membrane by washing twice at 37°C in 0.2 mol/L NaOH containing 1 mL/L sodium dodecyl sulfate (SDS) solution for 15 min and then re-hybridized with DIG-labeled probe for G3PDH.

Analysis of cytotoxicity

HepG2 2.2.15 cells were used for determining the cytotoxicity of PAP-S. Cells were plated onto a 96-well plate at a density of 1×10^4 cells per 100 µL prior to drug treatment. PAP-S was added at concentrations of 0.1, 1.0, 10, 50 and 100 µg/mL and the cells were refed with drug-containing fresh medium every 2 d for up to 4 d, and then 20 µL of 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl (MTT) was added to each well and further incubated in a CO₂ incubator at 37°C for 90 min. The absorbance (A) at 490 nm was read. Data were calculated as a percentage of negative control cells that were not treated with PAP-S.

Statistical analysis

Data points were obtained from at least three different cell cultures, in which each condition was assayed in triplicate. Values were expressed as mean \pm SD. To calculate the statistical significance of differences within or among groups, the paired *t*-test was used. Statistical significance was set at $P < 0.05$.

RESULTS

Anti-HBV activity of PAP-S in HepG2 2.2.15 cells

In HepG2 2.2.15 cells, MTT assay revealed that PAP-S had no inhibitory effect on cell proliferation up to 50 µg/mL. PAP-S inhibited the growth of HepG2 2.2.15 cells at concentrations above 50 µg/mL. The CD₅₀ value was 29.3 µg/mL.

As shown in Figure 1A-C, treatment of above 1 µg/mL PAP-S could statistically significantly reduce HBsAg, HBeAg and HBV DNA in a dose dependent manner as compared with vehicle controls ($P < 0.01$). The ID₅₀ of PAP-S to inhibit HBsAg and HBeAg production was 6.9 and 3.2 µg/mL on d 4, respectively. The therapeutic index (TI) for HBsAg and HBeAg was 4.3 and 9.3 on d 4, respectively. The data in this report clearly showed that the inhibitory activity of PAP-S on HBsAg and HBeAg was promoted time-dependently ($P < 0.01$). The suppression of HBV DNA was dose-dependent and approximately 53.3% inhibition was observed in the 4-d culture treated with PAP-S (10 µg/mL).

Effect of pXF3H-PAP on HBV DNA replication in cotransfected HepG2

On d 3 after transfection, levels of HBV core-associated DNA were detected by Southern blot. As shown in

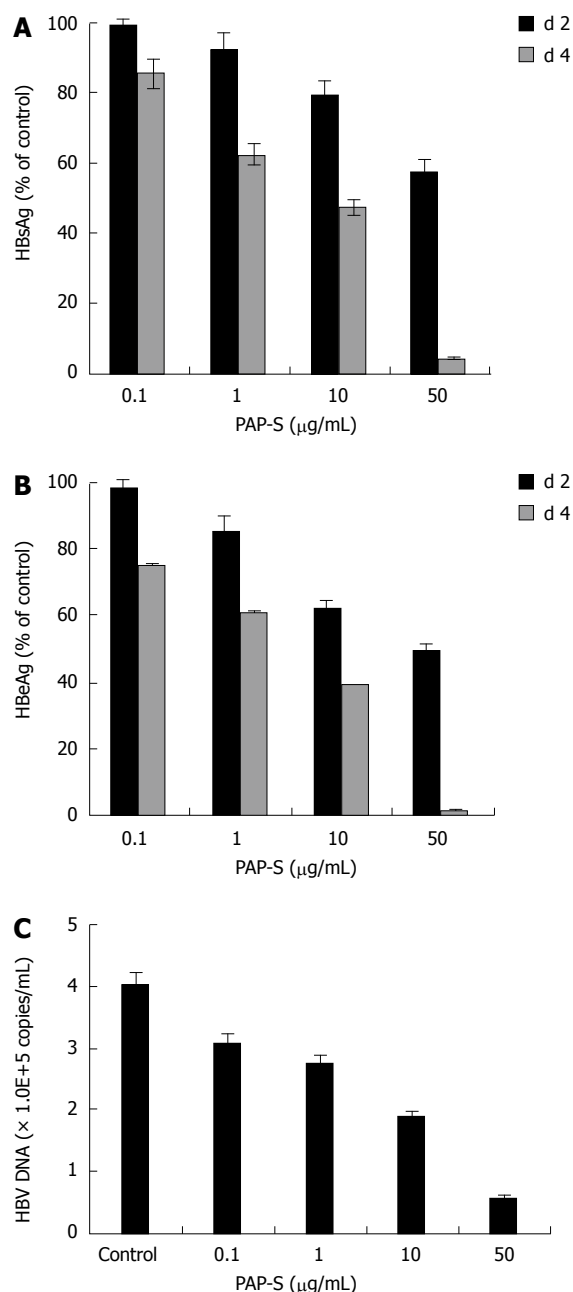


Figure 1 Effects of PAP-S on secreted HBsAg (A), HBeAg (B) and HBV DNA (C) in HepG2 2.2.15 cell cultures. Data are expressed as mean \pm SD of three independent experiments. Except the first group (0.1 µg/mL), $P < 0.01$ vs the corresponding controls (Student's *t*-test).

Figure 2, Western blot showed that PAP was expressed in cotransfected HepG2 cells. As compared with controls, the levels of HBV nucleocapside-associated DNA were reduced by 38.0% and 74.0% respectively after transfection of 1.0 and 2.0 µg plasmid pXF3H-PAP into HepG2 cells. The levels of HBV nucleocapside-associated DNA were reduced by 63.0% when the transfected HepG2 cells were exposed to 20.0 µmol/L 3TC (Figure 3). The present results indicated that PAP reduced the HBV nucleocapside-associated DNA in a dose-dependent manner.

Effects of pXF3H-PAP on secreted HBsAg and HBeAg in cotransfected HepG2

As compared with controls, the levels of HBsAg in the

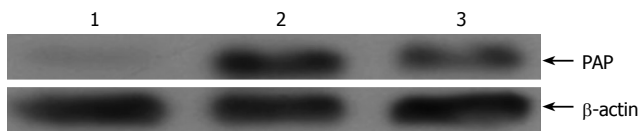


Figure 2 Western blot analysis of the expression of pXF3H-PAP in cotransfected HepG2 cells β -actin as internal reference; 1: pHBV1.3 (1.0 μ g) + pXF3H (2.0 μ g); 2: pHBV1.3 (1.0 μ g) + pXF3H-PAP (2.0 μ g); 3: pHBV1.3 (1.0 μ g) + pXF3H-PAP (1.0 μ g).

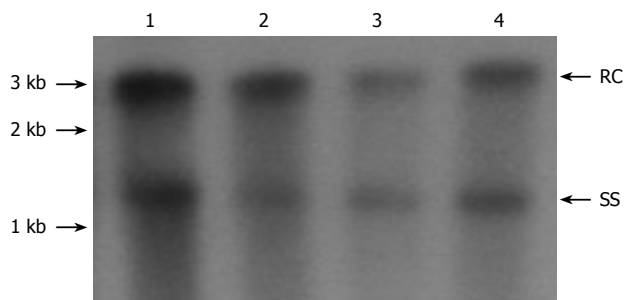


Figure 3 Southern blot analysis of the effect of pXF3H-PAP on intracellular HBV DNA replication in cotransfected HepG2 cells 3 d after transfection. RC: Relaxed circular DNA; SS: Single stranded DNA. 1: pHBV1.3 (1.0 μ g) + pXF3H (2.0 μ g) 2: pHBV1.3 (1.0 μ g) + pXF3H-PAP (1.0 μ g); 3: pHBV1.3 (1.0 μ g) + pXF3H-PAP (2.0 μ g); 4: pHBV1.3 (1.0 μ g) + 3TC (20.0 μ mol/L).

media were reduced by 76.8% and 99.7% respectively on d 3 after transfection of 1.0 μ g and 2.0 μ g plasmid pXF3H-PAP into HepG2 cells. The levels of HBsAg were reduced by 93.7% when the transfected HepG2 cells were exposed to 20.0 μ mol/L 3TC. The levels of HBeAg in the media were reduced by 72.7% and 99.3% respectively after transfection of 1.0 μ g and 2.0 μ g plasmid pXF3H-PAP into HepG2 cells. The levels of HBeAg were reduced by 82.4% when the transfected HepG2 cells were exposed to 20.0 μ mol/L 3TC. The present results indicated that PAP reduced the levels of HBsAg and HBeAg in a dose-dependent manner.

Effects of pXF3H-PAP on HBV core-associated RNA in cotransfected HepG2

Northern blot indicated that the HBV core-associated RNA levels of 3.5 kb, 2.4/2.1 kb in cotransfected HepG2 cells were significantly suppressed by PAP (Figure 4). After transfection of 2.0 μ g plasmid pXF3H-PAP, the levels of HBV core-associated RNA were reduced by 69% as compared with controls.

DISCUSSION

The anti-viral activity of PAP has been described against numerous pathogenic viruses^[3,4], which included poliovirus, HIV, herpes simplex virus, cytomegalovirus, influenza virus and now, the double-strand DNA virus, hepatitis B virus. The ability of PAP to inhibit viral protein synthesis and depurinate viral RNA and DNA^[10,11] as well as capped rRNA and mRNAs^[12] and its ability to inhibit ribosomal frame shifting and retransposition, make it an ideal candidate for anti-viral strategies.

In this study, we evaluated the effects of PAP from

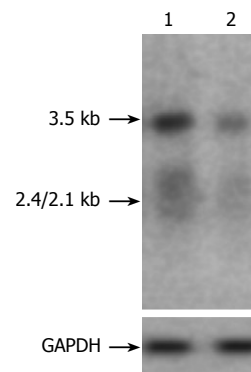


Figure 4 Northern blot analysis of the effect of pXF3H-PAP on intracellular HBV RNA transcription in cotransfected HepG2 cells on d 3 after transfection. GAPDH as internal reference; 1: pHBV1.3 (1.0 μ g) + pXF3H (2.0 μ g) 2: pHBV1.3 (1.0 μ g) + pXF3H-PAP (2.0 μ g).

PAP-S on hepatitis B virus *in vitro* by the HepG2.2.15 cell line transfected with hepatitis B virus DNA. Furthermore, we constructed the eukaryotic expression plasmid encoding PAP, cotransfected it and the replication competent wild-type plasmid pHBV1.3 into the HepG2 hepatoma cells in order to explore the inhibitory effect of PAP on HBV replication.

Previous studies suggested that the antiviral activity of PAP was attributed to its ability to inhibit protein synthesis by catalytically cleaving a specific adenine base from the highly conserved alpha-sarcin/ricin loop (SRL) of the large ribosomal RNA^[13]. However, animal studies have suggested that the antiviral action of PAP may not be contributed to its inactivation of ribosomes^[14,15]. PAP produced only 30% inhibition of total protein synthesis in herpes simplex virus-infected cells, whereas it inhibited virus production by 90%^[16]. Similarly, it has been reported that PAP inhibits HIV-1 production of p24 in both T cells and macrophages at concentrations that do not adversely affect protein synthesis^[17], which suggested that antiviral activity of PAP can be dissociated from its toxicity^[18-21].

Further research discovered that not only rRNA but also polynucleotide, single-stranded DNA, double-stranded DNA and mRNA, can be depurinated by PAP^[22,23]. Rajamohan^[11,24] treated HIV-1 RNA with various concentrations of PAP. The depurinating activity of test PAP was determined by measuring the amount of adenine and guanine released from the HIV-1 RNA using a quantitative HPLC method, which confirmed that PAP caused a concentration-dependent depurination from HIV-1 RNA. These findings indicate that PAP should be capable of recognizing and depurinating viral RNA.

In this study, it was found that both PAP-S and PAP encoded by a eukaryotic expression plasmid (pXF3H-PAP) could inhibit the levels of HBsAg, HBeAg and HBV DNA in a dose-dependent manner *in vitro*. At the RNA level, PAP might be capable of recognizing and depurinating HBV-mRNA, which included 3.5 kb pregenome mRNA and 2.4/2.1 kb pregenome mRNA, so that the levels of HBV mRNA were reduced. At the DNA level, on one hand, because the 3.5 kb pregenome mRNA was inhibited, the levels of HBcAg/HBeAg and HBV polymerase translated from 3.5 kb pregenome mRNA and the minus strand HBV DNA transcribed from the 3.5 kb pregenome mRNA template were reduced accordingly. On the other hand, PAP might be capable of recognizing and depurinating the new synthesized HBV DNA, so that the

levels of HBV DNA were reduced. At the protein level, on one hand, because HBV-mRNA was inhibited, the HBcAg and HBeAg translated from 3.5 kb mRNA and the HBsAg and pre-S antigen translated from 2.4 kb or 2.1 kb mRNA were inhibited accordingly. On the other hand, PAP might inhibit HBV protein translation by depurinating rRNA.

Further research demonstrated that PAP cleaved supercoiled pBR322 dsDNA, generating relaxed and linear molecules^[25]. PAP could identify the characteristic spatial conformation of supercoiled DNA and hydrolyze AMP in specific area, turn the supercoiled DNA into nicked circle DNA or linear DNA so that the supercoiled DNA exerted irreversible topological inactivation^[26]. HBV covalently closed circle (cccDNA) is also supercoiled DNA and has a characteristic spatial conformation, so we speculated that PAP could inhibit HBV by identifying and inactivating the HBV cccDNA template.

Several nucleoside analogs are under clinical development for use against HBV. Lamivudine (3TC), a nucleoside analog, and adefovir dipivoxil (ADV), an acyclonucleotide analog, are clinically approved. However, long-term treatment can induce viral resistance, and following the cessation of therapy, viral rebound is frequently observed^[27,28]. There continues to be a need for new antiviral agents with novel mechanisms of action. The anti-HBV mechanism of PAP might be different from 3TC that targeted the viral polymerase. A previous study had reported that PAP exhibited potent *in vivo* activities against nucleoside reverse transcriptase inhibitor-resistant HIV-1 in a surrogate human peripheral blood lymphocyte (Hu-PBL) SCID mouse model of human AIDS^[29]. More work is needed, however, to determine the mechanism of anti-HBV activity of PAP.

Moreover, we have detected the cytotoxicity of PAP on the cell and found that the tested concentration of PAP exerted little growth inhibition. The findings demonstrated that repetitive intravaginal administration of PAP at concentrations as high as 2000 times its *in vitro* anti-HIV IC₅₀ value was not associated with local or systemic toxicity^[30]. PAP may be useful as an anti-HBV agent.

In conclusion, the present study demonstrated that the eukaryotic expression plasmid pXF3H-PAP and PAP-S could effectively inhibit HBV antigen secretion and HBV replication in a dose-dependent manner *in vitro*. The present anti-HBV medicine cannot eliminate HBV cccDNA in the hepatocytes so that they cannot clear HBV thoroughly. PAP is therefore worthy to be further investigated as an excellent candidate for potential clinical studies.

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COMMENTS

Background

Hepatitis B virus (HBV) is a major cause of liver disease worldwide, ranging from acute and chronic hepatitis to cirrhosis and hepatocellular carcinoma. The anti-

HBV medicines such as interferon and nucleoside analogs are currently being implemented to treat affected patients. However, long-term treatment can induce viral resistance, and following the cessation of therapy, viral rebound is frequently observed. There continues to be a need for new antiviral agents with novel mechanisms of action.

Research frontiers

The aim of this study is to explore the inhibitory effects of Pokeweed antiviral protein (PAP) on HBV replication *in vitro*.

Innovations and breakthrough

This study clearly showed that PAP could effectively inhibit HBV replication and antigen expression in a dose-dependent manner *in vitro*. This is the first report of the anti-HBV effects of PAP.

Applications

PAP might be useful as a potential alternative or complementary anti-HBV agent. The perspective of future application: the further study for the exact mechanisms of anti-HBV activity of PAP.

Peer review

This is an interesting study. PAP has not been studied against HBV previously, so the manuscript provides new information to confirm that PAP, as expected, has an antiviral effect against HBV. The data and anti-HBV mechanisms are novel.

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RAPID COMMUNICATION

Effect of lifestyle intervention on non-alcoholic fatty liver disease in Chinese obese children

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CONCLUSION: Both a short-term lifestyle intervention and vitamin E therapy have an effect on NAFLD in obese children. Compared with vitamin E, lifestyle intervention is more effective. Therefore, lifestyle intervention should represent the first step in the management of children with NAFLD.

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Key words: Non-alcoholic fatty liver disease; Lifestyle intervention; Vitamin E; Obese; Children

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Abstract

AIM: To investigate the effect of lifestyle intervention on non-alcoholic fatty liver disease (NAFLD) in Chinese obese children.

METHODS: Seventy-six obese children aged from 10 to 17 years with NAFLD were enrolled for a one-month intervention and divided randomly into three groups. Group 1, consisting of 38 obese children, was an untreated control group without any intervention. Group 2, consisting of 19 obese children in summer camp, was strictly controlled only by life style intervention. Group 3, consisting of 19 obese children, received oral vitamin E therapy at a dose of 100 mg/d. The height, weight, fasting blood glucose (FBG), fasting serum insulin (FINS), plasma alanine aminotransferase (ALT), aspartate aminotransferase (AST), triglyceride (TG), total cholesterol (TCHO) and homeostasis model assessment-insulin resistance (HOMA-IR) were measured at baseline and after one month. All patients were underwent to an ultrasonographic study of the liver performed by one operator who was blinded to the groups.

RESULTS: The monitor indices of BMI, ALT, AST, TG, TCHO and HOMA-IR were successfully improved except in group 1. BMI and ALT in group 2 were reduced more significantly than in group 3 (2.44 ± 0.82 vs 1.45 ± 0.80 , $P = 0.001$; 88.58 ± 39.99 vs 63.69 ± 27.05 , $P = 0.040$, respectively).

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) tends to become epidemic in children worldwide in the latest decade. NAFLD is recognized as a cause of potentially progressive liver damage. The entire range of liver involvement in NAFLD can occur in children, such as hepatic macrovesicular steatosis without inflammation, steatosis with inflammation or fibrosis [which defined as non-alcoholic steatohepatitis (NASH)] and cirrhosis. NAFLD may be the hepatic aspect of the metabolic syndrome that comprises central obesity, insulin resistance, hypertension and dyslipidemia in adults and children^[1]. The natural history of NAFLD is only partly known, the disease slowly progresses to cirrhosis and the initial assessment of NASH as being benign is not supported by the available evidences^[2-8]. Insulin resistance has been shown to be the basic pathophysiological mechanism responsible for the fatty transformation of liver (first hit) as well as the second hit which leads to hepatocyte injury^[9,10]. The gold standard of diagnosis is liver biopsy, but it is not frequently performed in the pediatric population. Liver ultrasonography (US), although not sensitive enough to assess liver fibrosis or inflammation, has a sensitivity of 89% and a specificity of 93% for detecting histological steatosis^[11,12]. In the absence of liver

biopsy, presumed NASH is conventionally diagnosed by classical ultrasonography together with an elevated serum level of alanine aminotransferase (ALT)^[13].

Multiple therapeutic agents such as vitamin E, β -carotene, metformin, PPAR- γ agonists and the lipase inhibitor orlistat have demonstrated to be useful in NAFLD in a series of small cases^[14-18]. Lifestyle intervention (dietary restriction and exercise) has also improved the liver function of patients with NAFLD^[19]. Conflicting data on the therapeutic efficacy of these drugs have been reported in the literature^[14-18].

The aim of this study was to investigate the short-term effect of a lifestyle intervention on liver biochemistry and fasting insulin levels and compare with that of the vitamin E therapy in obese children with NAFLD.

MATERIALS AND METHODS

Seventy-six obese children, according to the criteria that a child was considered to be obese when the body mass index (BMI) exceeded the 95th BMI percentage for age and sex^[20], were enrolled in this study. The age of the subjects ranged from 10 to 17 years (mean 13.7 ± 1.9 years). They were all obese children with liver fatty infiltration in ultrasonic appearance and abnormal liver function with higher alanine aminotransferase (ALT) by at least 1.5 times over the upper normal limit which was diagnosed as NASH^[21]. They were divided randomly into three groups and the ultrasonography operator was blinded to the groups. Group 1 was an untreated control group, consisting of 38 obese children, who had not taken any medicine and lifestyle intervention. Group 2 had 19 obese children, taking no drug and treated only with strict lifestyle intervention at summer camp. Group 3, consisting of 19 obese children, was treated with vitamin E, while improving their behaviors and enjoying their lives freely by themselves at home. Patients who had positive markers for other liver diseases (hepatitis virus, TORCH, metabolic, genetic) or who had a history of alcohol intake were all excluded. Studies of the three groups were carried out at the same time, that of group 2 at the summer camp, and the others were done separately at home. They were all observed for one month, because the camp lasted only a month (Figure 1). The characteristics of the three groups are shown in Table 1.

Written informed consent was obtained from all participants and in case of minors, it was obtained from their parents. The study was conducted in accordance with the guidelines proposed in the Declaration of Helsinki and was approved by the Ethics Committee of the Children's Hospital of Zhejiang University School of Medicine.

Lifestyle intervention

Nineteen patients in group 2 were strictly controlled by lifestyle intervention without any drug therapy. They took part in the summer camp without their parents. The Nuote Nutrient Center which was in charge of the camp, consisted of nutrient experts, physical experts and pediatricians. Physical exercises, including swimming, playing basketball and tabletennis, were taken freely for three hours of aerobic exercise each

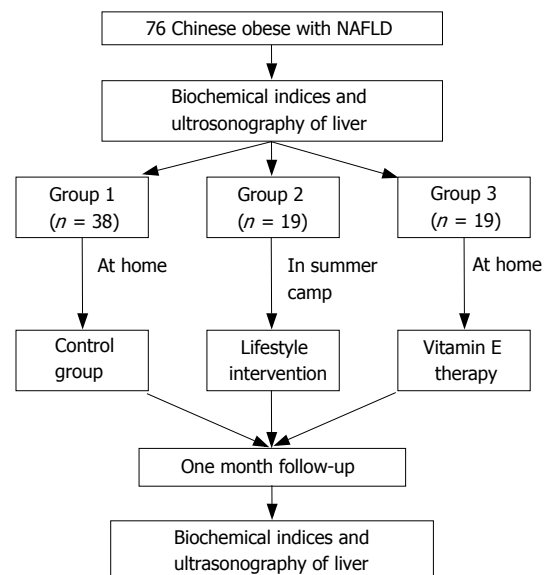


Figure 1 The dispositions of subjects. (76 obese children and adolescents with NAFLD aged between 10 and 17 years were enrolled in this study).

Table 1 Baseline characteristics of the three groups

Index	Group1 (n = 38)	Group2 (n = 19)	Group3 (n = 19)	F value	P value
Male/Female	26/12	13/6	13/6	0.000	1.000
Age (yr)	14.04 \pm 1.8	13.4 \pm 2.5	13.4 \pm 1.6	0.904	0.410
BMI (kg/m ²)	29.81 \pm 2.41	29.61 \pm 1.48	29.36 \pm 3.11	0.223	0.800
ZBMI	3.53 \pm 1.17	3.02 \pm 0.39	3.44 \pm 1.57	1.268	0.287
ALT (IU/L)	144.77 \pm 26.73	152.26 \pm 49.30	139.98 \pm 19.82	0.711	0.495
AST (IU/L)	86.63 \pm 21.54	93.26 \pm 38.94	78.55 \pm 23.11	1.432	0.245
TG (mmol/L)	1.44 \pm 0.35	1.38 \pm 0.38	1.51 \pm 0.33	0.698	0.501
TCHO (mmol/L)	4.70 \pm 1.18	4.82 \pm 0.91	4.61 \pm 1.03	0.200	0.819
FBG (mmol/L)	4.26 \pm 0.42	4.15 \pm 0.39	4.22 \pm 0.43	0.527	0.592
FINS (IU/L)	15.50 \pm 2.10	15.54 \pm 4.50	15.42 \pm 1.10	0.011	0.989
HOMA-IR	2.93 \pm 0.44	2.87 \pm 0.88	2.89 \pm 0.32	0.087	0.917

BMI: Body mass index; ALT: Plasma alanine aminotransferase; AST: Aspartate aminotransferase; TG: Triglyceride; TCHO: Total cholesterol; FBG: Fasting blood glucose; FINS: Fasting serum insulin; HOMA-IR: Homeostasis model assessment-insulin resistance; Group 1: Receiving no intervention as control; Group 2: Taking no drug and treated only with lifestyle intervention at summer camp; Group 3: Taking vitamin E, and controlled lifestyle intervention at home.

day. The diet management followed the principle of low-calorie [high in carbohydrate (50%) and low in fat (10%)] with the aim of a reduction in daily intake by 250 kcal. A total daily calorie intake was controlled from 1300 kcal to 1600 kcal based on the individual age. Two eggs and a bowl of soymilk were supplied at breakfast. Pork, egg, fish, shrimp, fresh vegetable, rice and corn were served at lunch and dinner. No beverage but mineral water was provided. They were requested to get up at 6:30 O'clock in the morning and take aerobic physical exercise in the morning and afternoon. In the evening they did their homework and watched TV for an hour, then went to sleep at 21:00 O'clock. The summer camp lasted one month. Nineteen patients in group 3 were controlled in lifestyle freely by themselves with total daily calorie intake and physical exercises (a low-

Table 2 Comparison of parameters among three groups by paired-samples *t* test

Index	Group	Before intervention	After intervention	<i>t</i> value	<i>P</i> value
BMI	Group1	29.81 ± 2.41	29.83 ± 2.32	-0.339	0.736
	Group2	29.61 ± 1.48	27.18 ± 1.83	12.892	0
	Group3	29.37 ± 3.11	27.92 ± 3.29	8.034	0
ZBMI	Group1	3.53 ± 1.17	3.55 ± 1.16	-1.765	0.086
	Group2	3.02 ± 0.39	2.15 ± 0.64	16.356	0
	Group3	3.44 ± 1.57	2.57 ± 1.57	9.438	0
ALT	Group1	144.77 ± 26.73	144.82 ± 25.51	-0.076	0.94
	Group2	152.26 ± 49.30	63.68 ± 23.38	9.654	0
	Group3	139.97 ± 19.82	73.28 ± 10.11	13.219	0
AST	Group1	86.63 ± 21.54	85.73 ± 19.60	1.017	0.316
	Group2	93.26 ± 38.94	45.09 ± 19.18	6.699	0
	Group3	78.55 ± 23.11	45.80 ± 6.66	6.9	0
TG	Group1	1.44 ± 0.35	1.46 ± 0.31	-0.69	0.494
	Group2	1.38 ± 0.38	0.99 ± 0.37	3.851	0.001
	Group3	1.51 ± 0.33	1.27 ± 0.28	4.6	0
TCHO	Group1	4.70 ± 1.18	4.69 ± 1.09	0.204	0.84
	Group2	4.83 ± 0.92	4.54 ± 0.98	2.783	0.012
	Group3	4.61 ± 1.03	4.23 ± 0.82	2.24	0.038
FBG	Group1	4.26 ± 0.42	4.26 ± 0.32	-0.046	0.964
	Group2	4.15 ± 0.39	4.13 ± 0.42	0.154	0.879
	Group3	4.22 ± 0.43	4.08 ± 0.41	1.11	0.279
FINS	Group1	15.50 ± 2.10	15.71 ± 2.19	-0.941	0.353
	Group2	15.54 ± 4.50	8.53 ± 4.08	4.322	0
	Group3	15.42 ± 1.10	8.77 ± 2.46	10.26	0
HOMA-IR	Group1	2.93 ± 0.44	2.97 ± 0.51	-0.886	0.382
	Group2	2.87 ± 0.88	1.63 ± 0.92	3.579	0.002
	Group3	2.89 ± 0.32	1.62 ± 0.59	8.08	0

BMI: Body mass index; ALT: Plasma alanine aminotransferase; AST: aspartate aminotransferase; TG: Triglyceride; TCHO: Total cholesterol; FBG: Fasting blood glucose; FINS: Fasting serum insulin; HOMA-IR: Homeostasis model assessment-insulin resistance.

intensity aerobic exercise to reach a 50%-60% maximum of their heart beat and maintained for 30 min, 2-3 times a week). Thirty-eight patients in group 1 did not receive any lifestyle intervention.

Drug therapeutic protocol

Patients in group 3 received vitamin E capsule at a dose of 100 mg/d for one month. Groups 1 and 2 received no drug treatment.

Monitoring indices

Indices of all patients, including the height, weight, fasting blood glucose (FBG), fasting serum insulin (FINS), plasma alanine aminotransferase (ALT), aspartate aminotransferase (AST), triglyceride (TG) and total cholesterol (TCHO), were measured in the morning after an overnight fast and repeated after a month. Fasting blood glucose was measured by the glucose oxidase method (Beijing North Biotechnology Invest, China) with intra-assay and inter-assay CV of 2.1% and 4.4%, respectively. Fasting serum insulin levels were determined by radioimmunity assay (Beijing North Biotechnology Invest, China). The intra-assay and inter-assay CV were 6.4% and 9.7%, respectively. ALT, AST, TG and TCHO were measured by routine laboratory test (BECKMAN Synchron Clinical System CX4, American) at the central laboratory of our unit. In this study, hypertransaminasemia (or elevation of serum ALT) was defined as serum ALT levels being raised by

at least 1.5 times over the upper normal limit (normal range: < 50 IU/L). BMI = weight (kg)/height (m²). To compare BMI among different ages in both boys and girls, BMI Z-score was considered. The Z-score represents the number of s.d. above or below the mean value based on standardized tables for children^[22]. Insulin resistance was assessed by homeostasis model assessment (HOMA-IR) based on serum fasting insulin and glucose concentrations. HOMA-IR = (FINS in mIU/L × FBG in mmol/L)/22.5. Our previous study proved that HOMA-IR was a valid insulin sensitivity index from OGTT parameters in obese children and adolescents^[23].

All patients underwent an ultrasonographic study of the liver performed by one operator who was blinded to the groups. The apparatus used (LOGIC 500, GE corporation) was equipped with a convex 3.0-5.0 MHz probe. Longitudinal, subcostal, ascending, and oblique scans were performed. The ultrasonographic criteria of liver-kidney echo discrepancy, echo penetration into the deep portion of the liver, and clarity of liver blood vessel structures were used to diagnose fatty liver according to Graif M, *et al*^[24].

Statistical analysis

Statistical analyses were conducted using SPSS software (Version 13.0). Pearson Chi-square was used to measure the enumeration data between subgroups. Quantitative data were presented as mean ± SD or median (range) and estimated by one-way ANOVA or paired-samples *t* test. Mann-Whitney test was used to evaluate the significance of skewed distributed data. A two-tailed *P* < 0.05 was considered statistically significant.

RESULTS

A total of 76 NASH patients were analyzed and observed for a month. The baseline characteristics of the three groups were not significantly different (*P* > 0.05) (Table 1). After one month, these characteristics in group 1 were not improved (*P* > 0.05) (Table 2).

In groups 2 and 3, all patients had declined BMI, Z_{BMI}, ALT, AST, TG, TCHO, FINS and HOMA-IR after a month. There was more significant reduction in BMI and ALT levels after intervention in group 2 than in group 3 (*U* = 73.000, *P* = 0.001; *U* = 117.000, *P* = 0.040, respectively). No difference was found in the other indices between groups 2 and 3 (*P* > 0.05). Ten patients (52.63%) in group 2 had normal liver functions after the camping was completed.

Nine patients (47.37%) in group 3 became normal in liver functions in the end. The liver ultrasonography did not demonstrate any predominant changes after a one-month lifestyle intervention.

DISCUSSION

Non-alcoholic fatty liver disease affects a large proportion of the population. In our previous study, the prevalence of NAFLD was 22.41% among all obese subjects and the male is apt to obesity with NAFLD^[1,25]. The pathogenesis of NAFLD has remained poorly understood since the earliest

description of this disease. However, insulin resistance and oxidative stress play critical roles in the pathogenesis of non-alcoholic fatty liver disease. As yet no accepted drug treatment of NAFLD/NASH has been reported.

In this study, we found that the body weights of all the patients except control group were successfully reduced. Lifestyle intervention was associated with ALT improvement after a month. And it seemed that vitamin E therapy also improved the liver function. At the same time, lifestyle intervention proved to be more efficient than vitamin E therapy on BMI and ALT. The liver ultrasonography did not demonstrate any predominant change after a month intervention. It is likely that ultrasonography is not sensitive enough to detect an initial and short improvement in steatosis. Weight reduction will improve not only the liver condition but also the metabolic (insulin resistance) syndrome. Exercise is known to improve the sensitivity of muscle mass to insulin^[26-28]. Furthermore, a recent clinical trial showed that exercise has modest therapeutic effect in reducing visceral fat and improving glucose intolerance^[29]. These may partially explain the beneficial effect of lifestyle intervention in improving the hypertransaminasemia. Frequency and intensity of ideal exercise for treatment of NAFLD remains unknown. Suzuki A *et al* reported that patients with NAFLD should be encouraged to keep regular exercise and at least twice a week^[30].

Vitamin E is frequently used among patients with NAFLD. The useful effect of vitamin E on inflammation and fibrosis among patients with NASH has been attributed to its potent antioxidant action. Oxidant stress has been cited as an important second hit in the pathogenesis of NASH, and obese children have been demonstrated to have significantly decreased serum levels of α -tocopherol^[31-34]. In these studies, the dose of vitamin E was 300-1200 mg/d. In one study, vitamin E 300 mg/d was associated with fibrosis reversal^[35]. However, Nobili *et al* reported that in 90 patients in biopsy-proven NAFLD children, a balanced calorie diet, physical exercise, and placebo or alpha-tocopherol 600 IU/d plus ascorbic acid 500 mg/d and a 12-mo double-blind placebo study found that vitamin E therapy had no effect on ALT or insulin resistance^[36]. Kugelmans *et al* found that vitamin E improved insulin sensitivity and several of its associated parameters, including ALT levels in overweight otherwise healthy subjects but the effect of treatment was not sustained^[37]. In our study, vitamin E was also found to improve insulin sensitivity and ALT level in a month therapy. So, we conclude that a short-time therapy with 100 mg vitamin E and lifestyle intervention may have an effect on ALT levels and insulin resistance in children with NAFLD.

Lifestyle intervention has been recommended for the treatment of NAFLD in obese children. Early intervention should attempt to increase physical activity while implementing dietary and other antiobesity measures. The emphasis should be laid on slow and modest reduction of body mass, not exceeding 2 pounds (1 kg)/wk, coupled with increased physical activity^[20]. It is very encouraging that lifestyle intervention had short-term effect, but the long-term effect on NAFLD remains to be clarified. After the camping, the children and their monitors were assembled

and taught how to do exercise and how to arrange daily diet. They were encouraged to have a low-intensity aerobic exercise such as playing basketball and table-tennis, quick walking, slow running or string jumping, etc to reach a 50%-60% maximum of their heart beat and maintain for at least 30 min 2-3 times a week. Diet was tailored based on individual preferences and balanced as hypocaloric diet (25-30 cal/kg per day, carbohydrate 50%-60%, fat 23%-30%, protein 15%-20%, fatty acid: two-thirds saturated, one-third unsaturated, $\omega 6/\omega 3$ ratio = 4:1).

In summary, our results suggest that simple lifestyle intervention with physical exercise and diet in children with NAFLD can lead to a significant improvement of liver function and insulin resistance. A short-term vitamin E therapy also has a effect on NAFLD in obese children. Compared with vitamin E therapy, lifestyle intervention is more effective. Therefore, lifestyle intervention should represent the first step in the management of children with NAFLD.

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COMMENTS

Background

Non-alcoholic fatty liver disease (NAFLD) is likely to become epidemic in children worldwide in the latest decade. NAFLD is recognized as a cause of potentially progressive liver damage. The gold standard of diagnosis is liver biopsy but it is not frequently performed in the pediatric population. In the absence of liver biopsy, presumed NASH is conventionally diagnosed by classical ultrasonography together with an elevated serum level of alanine aminotransferase (ALT).

Research frontiers

Multiple therapeutic agents such as vitamin E, metformin, and lifestyle intervention have demonstrated to be useful in NAFLD. However, no accepted drug treatment of NAFLD/NASH has been reported.

Innovations and breakthroughs

Although the relation between diet and fat liver is known, it is the first report about lifestyle intervention on NAFLD in Chinese obese children. In this study, both a short-term lifestyle intervention and a short-term vitamin E therapy have an effect while lifestyle intervention is more effective than vitamin E.

Applications

Lifestyle intervention should represent the first step in the management of children with NAFLD.

Terminology

NAFLD encompasses a spectrum of disease ranging from simple hepatic steatosis to non-alcoholic steatohepatitis. The entire range of liver involvement characterizing NAFLD can occur in children, such as hepatic macrovesicular steatosis without inflammation, steatosis with inflammation or fibrosis (which defined as non-alcoholic steatohepatitis, NASH), and cirrhosis. NAFLD may be the hepatic aspect of the metabolic syndrome that comprises the central obesity, insulin resistance, hypertension and dyslipidemia in adults and children.

Peer review

This manuscript is of some interest because therapeutic intervention is limited to one month and the adherence to the program is really rigorous. This experiment may provide insights on the short-term modification of some indexes of NAFLD that probably anticipate the improvement of fatty liver.

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A pilot study on combination of cryosurgery and ^{125}I iodine seed implantation for treatment of locally advanced pancreatic cancer

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Abstract

AIM: To study the therapeutic value of combination of cryosurgery and ^{125}I iodine seed implantation for locally advanced pancreatic cancer.

METHODS: Forty-nine patients with locally advanced pancreatic cancer (males 36, females 13), with a median age of 59 years, were enrolled in the study. Twelve patients had liver metastases. In all cases the tumors were considered unresectable after a comprehensive evaluation. Patients were treated with cryosurgery, which was performed intraoperatively or percutaneously under guidance of ultrasound and/or computed tomography (CT), and ^{125}I iodine seed implantation, which was performed during cryosurgery or post-cryosurgery under guidance of ultrasound and/or CT. A few patients received regional celiac artery chemotherapy.

RESULTS: Thirteen patients received intraoperative cryosurgery and 36 received percutaneous cryosurgery. Some patients underwent repeat cryosurgery. ^{125}I iodine seed implantation was performed during freezing procedure in 35 patients and 3-9 d after cryosurgery in 14 cases. Twenty patients, 10 of whom had hepatic

metastases received regional chemotherapy. At 3 mo after therapy, CT was repeated to estimate tumor response to therapy. Most patients showed varying degrees of tumor necrosis. Complete response (CR) of tumor was seen in 20.4% patients, partial response (PR), in 38.8%, stable disease (SD), in 30.6%, and progressive disease (PD), in 10.2%. Adverse effects associated with cryosurgery included upper abdomen pain and increased serum amylase. Acute pancreatitis was seen in 6 patients one of whom developed severe pancreatitis. All adverse effects were controlled by medical management with no poor outcome. There was no therapy-related mortality. During a median follow-up of 18 mo (range of 5-40), the median survival was 16.2 mo, with 26 patients (53.1%) surviving for 12 mo or more. Overall, the 6-, 12-, 24- and 36-mo survival rates were 94.9%, 63.1%, 22.8% and 9.5%, respectively. Eight patients had survival of 24 mo or more. The patient with the longest survival (40 mo) is still living without evidence of tumor recurrence.

CONCLUSION: Cryosurgery, which is far less invasive than conventional pancreatic resection, and is associated with a low rate of adverse effects, should be the treatment of choice for patients with locally advanced pancreatic cancer. ^{125}I iodine seed implantation can destroy the residual surviving cancer cells after cryosurgery. Hence, a combination of both modalities has a complementary effect.

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Key words: Pancreatic cancer; Cryosurgery; Cryoablation; ^{125}I iodine seed implantation

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INTRODUCTION

Pancreatic cancer is a rapidly growing tumor that is nearly always fatal. The majority of pancreatic cancers are detected at a late stage of illness, and only a minority of patients are candidates for curative surgical resection. Overall, the 1- and 5-year survival rates are only 20% and 5%, respectively^[1-3]. Paclitaxel and gemcitabine are considered to be effective agents in pancreatic cancer, but their response rates are no more than 20%, and the effectiveness is less than 6 mo^[4,5]. Therefore, it is necessary to seek novel treatment modalities^[6,7]. This report examines the role of combined cryosurgery and ¹²⁵I seed implantation in the treatment of locally advanced pancreatic cancer.

MATERIALS AND METHODS

Patients

From March 2001 to November 2007, forty-nine patients with locally advanced pancreatic cancer underwent cryosurgery combined with ¹²⁵I seed implantation. There were 36 males and 13 females, aged 28-89 years, with a median age of 59 years. Tumor size ranged from 2.2-7.1 cm in the largest diameter. Twelve patients had hepatic metastasis. In all patients, the diagnosis was based on ultrasound, computed tomography (CT) and MRI imaging, and 38 patients had a positive histology. Before hospitalization, 14 cases had received 4-6 cycles of chemotherapy (gemcitabine, cisplatin, 5-FU). All patients received a comprehensive evaluation and were considered to be unresectable. The patients were provided information on cryosurgery guidelines, and the study received ethical approval.

Cryosurgery

Cryosurgery was performed with intraoperative or percutaneous approaches. Intraoperative cryosurgery: Patients were administered general anesthesia and were positioned for an upper abdominal incision. The involved pancreas was exposed by trans-peritoneal mobilization of the bowel and stomach. Once the pancreatic mass was identified, an 18-gauge Tru-Cut biopsy needle was used to obtain one or two cores of tissue from the solid mass. If it was determined that the tumor was unresectable, after a thorough investigation, cryosurgery was performed under direct vision and under ultrasound guidance. A variable number (one to three) of 2 or 3 mm cryoprobes were placed directly into the pancreatic mass and positioned under ultrasound guidance. In general, lesions smaller than 3 cm could be frozen reliably with a single centrally placed 3-mm probe, whereas larger lesions required multiple probes. A double cycle of freeze/thaw procedure was used with an argon gas-based cryosurgical unit (EndoCare, Inc., CA, USA). Each cryoprobe was cooled to -160°C and the resulting iceball was monitored with ultrasound until the frozen region encompassed the entire mass of the tumor with at least a "0.5-cm safe border". The tissue was then allowed to slowly thaw to 0°C. A second cycle of freezing/thawing was performed after repositioning of the cryoprobes. The cryoprobes were then removed and the

still-frozen tract made by the cryoprobe was packed with thrombin-coated Gelfoam to control bleeding. Metastases of the liver were treated with cryosurgery at the same time^[8,9].

Percutaneous cryosurgery: The procedure was performed under local anesthesia and under guidance of ultrasound or CT. Based on the location of the tumor, cryoprobe insertion was often carried out via the retroperitoneal approach. Generally, 2 or 3 mm cryoprobes were used. For tumors greater than 3 cm in size, 2 to 3 probes were used. For liver metastases, simultaneous cryosurgery was performed using additional cryoprobes which were inserted through the right intercostal space. The cryosurgery procedure was similar to that performed intraoperatively^[9].

Seed implantation: The procedure was performed either at the time of cryosurgery or after cryosurgery through the percutaneous approach under ultrasound or CT guidance. The ¹²⁵I seeds were implanted at the tumor border. The number of seeds employed depended on the tumor size, with each seed implanted at a distance of 0.5 cm.

Postoperative management: The patients were instructed to stop eating for at least 3 d after the procedure. An analogue of somatostatin was given by intravenous infusion, usually for 3-4 d, or extended further until the abdominal pain subsided and the elevated serum amylase levels normalized. Aprotinin (Trasylol), an inhibitor of pancreatic enzymes, and a proton pump inhibitor were given by intravenous infusion to patients with abdominal pain and elevated serum amylase levels.

Adjuvant regional chemotherapy: Infusion of chemotherapeutic drugs was initiated one wk after cryosurgery, via a catheter in the celiac artery or hepatic artery. The treatment consisted of cycles of 5-FU 500 mg/m², mitomycin C 8.5 mg/m² and gemcitabine 500 mg/m², every 2 wk.

Follow-up

Postoperative follow-up was performed at one mo after treatment and every 3 mo thereafter. On each visit, the patients were assessed by tumor marker assay, abdominal ultrasonography, and CT. Some patients were examined by positron emission tomography-CT PET-CT. The efficacy of cryosurgery was evaluated based on tumor size and survival of the patients. Changes in tumor mass were measured according to The Response Evaluation Criteria in Solid Tumors (RECIST) protocol^[10], which is based on objective measurements of the tumor size before and after treatment. Complete response (CR) means that all targeted lesion had disappearance (scar) or reduced to less than 25% of the original size. Partial response (PR) means a greater than 30% decrease in the sum of the largest diameter of all targeted lesions. Stable disease (SD) means less than 30% decrease in the sum of the largest diameter of all targeted lesions. Progressive disease (PD) means an increase of greater than 20% in the sum of the largest diameter of all targeted lesions.

All radiologic studies were reviewed by the same radiologist with an expertise in pancreatic imaging. Ultrasound-guided biopsy was performed for lesions that were suspicious for recurrence. Cryosurgery was repeated if histology showed a positive result. The presence of a persistent nodule on imaging studies without tumor activity on PET-CT, with decreasing or normal tumor markers (CA19-9), or no changes in the absence of any other treatment for an interval of at least 6 mo after cryosurgery, was considered as remnant tumor. Tumor recurrence was determined by a positive histology, or by the combination of an increase in the cryotreated lesion on ultrasound, CT or PET-CT imaging, an increase in the tumor markers or by the discovery of metastases.

Statistical analysis

Survival was calculated using the Kaplan-Meier test^[11]. Prognostic factors influencing survival were tested using the Log-rank, Tarone-Ware or Breslow test for univariate analysis and Cox regression^[12]; Cox's proportional hazard model with the forward-stepwise method (likelihood ratio) was used for multivariate analysis with various covariates. A significant difference was indicated by $P < 0.05$. Statistical analysis was performed using SPSS version 11.5 (SPSS, Chicago, USA).

RESULTS

Thirteen patients received intraoperative cryosurgery, and 36 underwent percutaneous cryosurgery. Among the patients who received percutaneous cryosurgery, 17 received a second course of cryosurgery and 3 received three courses of cryosurgery. ¹²⁵Iodine seed implantation was performed during cryosurgery in 35 patients, and 3-9 d after cryosurgery in 14 cases. The median number of ¹²⁵Iodine seeds implanted was 34, with a range of 18-54 seeds. Twenty patients received adjuvant regional chemotherapy, 10 of whom had hepatic metastases. Five patients received 1 cycle of chemotherapy, ten received 2 cycles, three 3 cycles and two 4 cycles.

Response to treatment: Based on CT findings, at 3 mo after treatment, most patients showed varying degrees of tumor necrosis. The results of CR, PR, SD and PD were 20.4% (10/49), 38.8% (19/49), 30.6% (15/49) and 10.2% (5/49), respectively.

Adverse reactions: As shown in Table 1, 69.4% of patients had abdominal pain, which usually subsided in 2-3 d. About one-half of the patients (51.0%) had elevated serum amylase levels, which generally ranged 1-2 times of the normal reference values and lasted for 5-7 d. Acute pancreatitis with acute abdominal pain, and elevated serum amylase levels to four times or more was seen in 6 patients (12.2%), one of whom developed severe pancreatitis with intra-abdominal fluid effusion, and serum amylase levels 12 times of the normal reference values. All patients with pancreatitis recovered with conservative management. Three patients (6.1%) had intra-abdominal bleeding, however, abdominal fluid obtained by paracentesis did

Table 1 Adverse effects of pancreatic cryosurgery

Adverse effects	No. of patients (n)	%
Abdominal pain	34	69.4
Fever	26	53.1
Acute pancreatitis ¹	6	12.2
Increased amylase levels ²	25	51.0
Abdominal bleeding ³	3	6.1
Pulmonary infection	3	6.1
Myocardial infarction	1	2.0
Cerebral infarction	1	2.0

¹With abdominal pain and fever; ²Including 6 patients with acute pancreatitis;

³With abdominal pain.

not have increased amylase levels. The intra-abdominal bleeding disappeared within four days. Nearly one-half of the patients (53.1%) had fever of 38-39.5°C, accompanied with chills. Fever persisted for 3-4 d, generally less than 7 d. Two patients had pulmonary infection, which recovered within 7-10 d with antibiotic therapy. Two patients aged 78 and 91 years, developed cerebral infarction and myocardial infarction respectively. There was no treatment-related mortality.

Overall survival

During a median follow-up of 18 mo (range of 5-40 mo), the median duration of survival was 16.2 mo. Twenty-six patients (53.1%) survived 12 mo or more, 8 of whom lived for 24 mo or more. The patient with the longest survival (40 mo) is still living without any evidence of tumor recurrence. A total of 36 patients died, in whom 17 died of cancer spread, 11 with hepatic metastases died of liver failure, 5 of cardio-cerebral vascular diseases and 3 of unknown causes. The 6-, 12-, 24- and 36-mo survival rates were 94.9%, 63.1%, 22.8% and 9.5% respectively (Figure 1A).

Univariate analysis was performed for factors influencing survival. Of the 5 variables tested, adjuvant chemotherapy and hepatic metastases were associated with a poor prognosis. The mode of cryosurgery (intra-operative *vs* percutaneous), tumor size (≤ 4 cm *vs* > 4 cm), and location (head *vs* body or tail) did not show independent significance for prognosis (Figure 1A and B).

The univariate analysis (Breslow test) of median survival in the different subgroups of patients with pancreatic cancer is shown in Table 2. The following factors were associated with longer median survival: cancer of pancreatic head, absence of hepatic metastases and absence of adjuvant chemotherapy.

A Cox model for multivariate regression analysis showed that apart from adjuvant chemotherapy, of the six factors tested, including patient's age, gender, tumor size, location, mode of cryosurgery, number of ¹²⁵Iodine seeds implanted and hepatic metastases, only hepatic metastases was an independent prognostic factor ($P = 0.007$).

Six case studies

Case 1. Male, 80 years old. Ultrasound showed a 3 cm \times 3 cm lesion in the pancreatic neck. Biopsy revealed cystadenocarcinoma. The patient underwent percutaneous cryosurgery with ¹²⁵Iodine seed implantation under CT

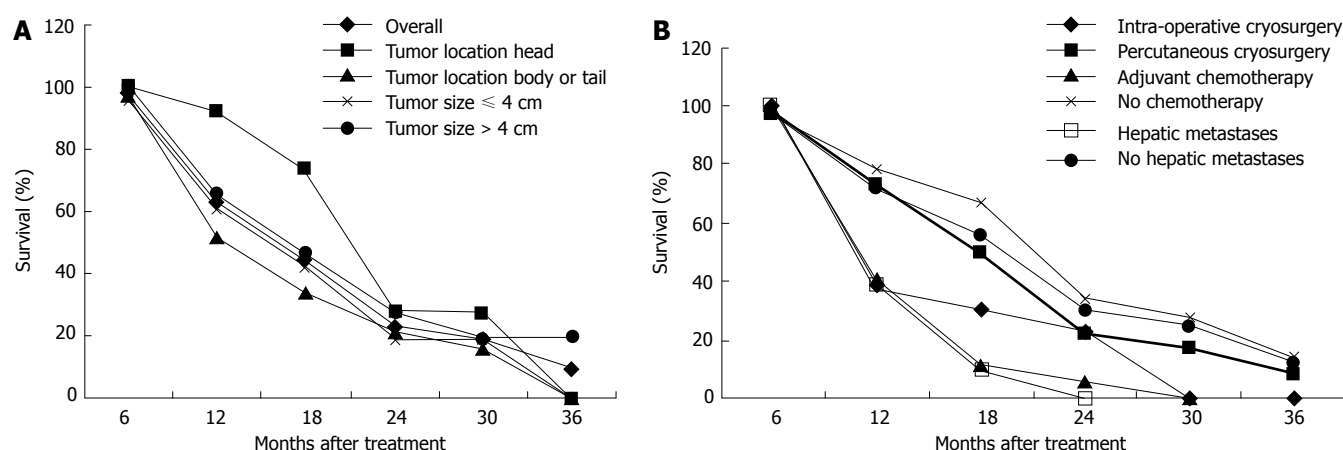


Figure 1 Survival rates of patients with pancreatic cancer. **A:** Overall results and those in patients with different location and size of the tumor; **B:** Pancreatic cancer with different modes of therapies, and with or without hepatic metastases.

guidance. Three mo after treatment, CT scan showed tumor necrosis, containing ^{125}I iodine particles. Current ultrasound and CT scan show that the original tumor has decreased to 1.5 cm × 1.1 cm in size (Figure 2). The patient has had recurrence-free survival of 40 mo.

Case 2. Male, 61 years old. CT scan showed low-density areas, 4 cm × 5.5 cm in size in the body of pancreas and 3 intrahepatic lesions ranging from 2 cm to 5 cm in size. Biopsy showed adenocarcinoma. The serum CA19-9 was 512 IU. The patient underwent percutaneous cryosurgery and ^{125}I iodine seed implantation under CT/ultrasound guidance for the pancreatic lesion and hepatic metastases. Repeat CT scan showed tumor shrinkage and stability of lesions in both the pancreas and liver (Figure 3). Ultrasound-guided biopsy showed no evidence of cancer. CA19-9 levels decreased to < 40 IU. The patient is now alive for 27 mo.

Case 3. Male, 36 years old. Ultrasound and CT revealed a mass in the pancreatic head with dilated common bile duct. Serum CA19-9 was 210 IU. The patient underwent laparotomy which revealed a mass, 5 cm × 5 cm in size in the pancreatic head. Biopsy showed moderately differentiated adenocarcinoma. A palliative cholecystojejunostomy was carried out to relieve the obstructive jaundice, and cryosurgery was performed under direct vision and ultrasound guidance. A repeat CT at three mo after treatment showed shrinkage and necrosis of the pancreatic mass with “honeycomb”-like change (Figure 4). CA19-9 had decreased to 48 IU. The patient survived for 19 mo.

Case 4. Male, 67 years old, with obstructive jaundice was found to have a mass, 5 cm × 3 cm in size in the pancreatic head with dilated common bile duct and gallbladder. Biopsy of the mass showed moderately differentiated mucinous adenocarcinoma. He was treated with percutaneous cryosurgery and ^{125}I iodine seed implantation. CT at 8 mo after treatment showed shrinkage and necrosis of the pancreatic mass (Figure 5).

Case 5. Female, 59 years old. CT scan showed a mass, 4 cm × 3 cm in size, in the pancreatic tail. Biopsy revealed adenocarcinoma. Percutaneous cryosurgery with ^{125}I iodine seed implantation was performed (Figure 6). Follow-up

Table 2 Median survival in different subgroups of patients with pancreatic cancer (breslow test)

Patient subgroups	n	Median survival (mo)	P
Tumor location			
Pancreatic head	15	22	0.0204
Pancreatic body or tail	34	12	
Tumor size			
≤ 4 cm	24	13	0.7425
> 4 cm	25	14	
Mode of cryosurgery			
Inoperative	13	11	0.1907
Percutaneous	36	14	
Adjuvant chemotherapy			
Yes	20	11	0.0006
No	29	22	
Hepatic metastases			
Yes	12	11	0.0088
No	37	19	

after 14 mo of treatment showed stable pancreatic tumor. The patient is currently alive 28 mo after diagnosis.

Case 6. Female, 59 years old. Ultrasound and CT showed a mass of the pancreatic head, 4 cm × 4 cm in size. Biopsy showed poor-differentiated adenocarcinoma. She underwent percutaneous cryosurgery and ^{125}I iodine seed implantation. Twelve mo later, the tumor in the pancreatic head was stable, however a new lesion has appeared in the pancreatic body. The patient underwent a second course of percutaneous cryosurgery for the lesion in the pancreatic body. Follow-up PET-CT at 3 mo after the treatment, showed a significant decrease in the metabolic activity of the original lesion (Figure 7).

DISCUSSION

Cryosurgery has provided a novel therapeutic approach to the treatment of benign and malignant tumors, especially unresectable tumors^[13]. A number of clinical trials have been published using this modality for the treatment of liver cancer, prostate cancer, kidney tumors, and breast cancer, with encouraging results^[14,15].

There are few reports on the use of cryosurgery for the treatment of pancreatic cancer. Kovach^[16] reported 9



Figure 2 Pancreatic CT scan in case 1. **A:** Before treatment; **B:** Three months after treatment; **C:** Twelve months after treatment.



Figure 3 CT scan of case 2. **A:** Before treatment, a mass was seen in pancreatic body and tail; **B:** One month after treatment; **C:** Six months after treatment.

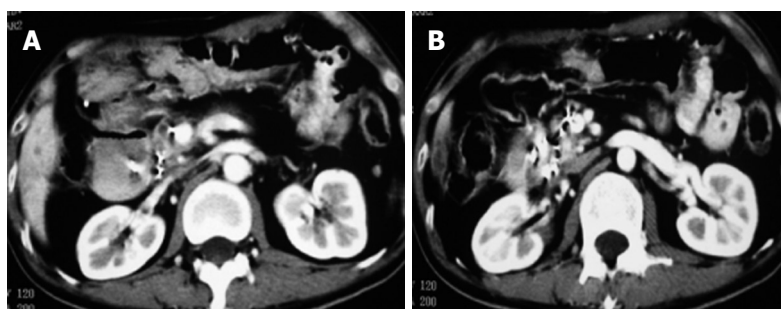


Figure 4 CT scan in case 3. **A:** One month after treatment; **B:** Three months after treatment.

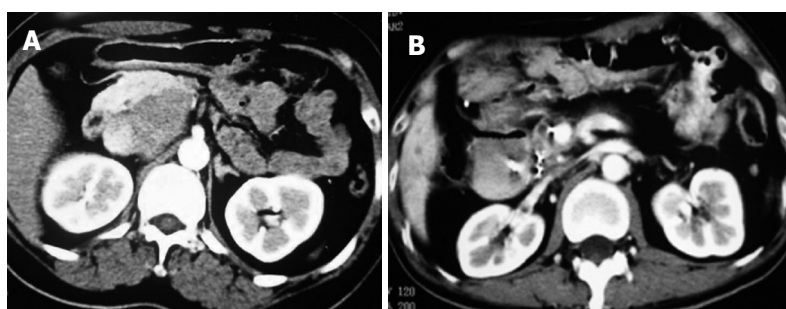


Figure 5 CT scan in case 4. **A:** Before treatment; **B:** Eight months after treatment.

patients with unresectable pancreatic cancer who received a total of 10 sessions of intraoperative cryosurgery under ultrasound guidance. There was no cryosurgery-related mortality and no post-cryosurgery pancreatic fistulae or pancreatitis. Following treatment, patients experienced alleviation of pain and reduction in the use of analgesic agents. All patients were able to take normal diet at the time of discharge from the hospital. Patiutko^[17] treated

30 patients with locally advanced pancreatic cancer with a combination of cryosurgery and radiation. All patients had effective control of pain, reduction in CA19-9, improvement of performance, and increase in the survival rate. Korpan^[18] summarized the experience of cryosurgery for pancreatic cancer, and concluded that most patients obtained good results with this therapeutic modality.

The effectiveness of cryosurgery is dependent upon

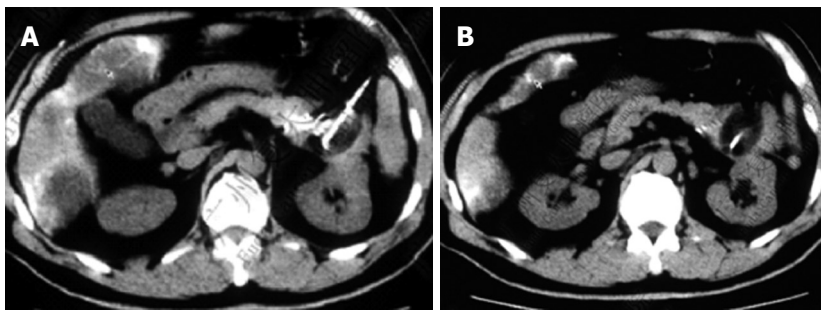


Figure 6 CT scan in case 5. **A** and **B** showing cryoprobe and ^{125}I iodine seeds in different layers during treatment.

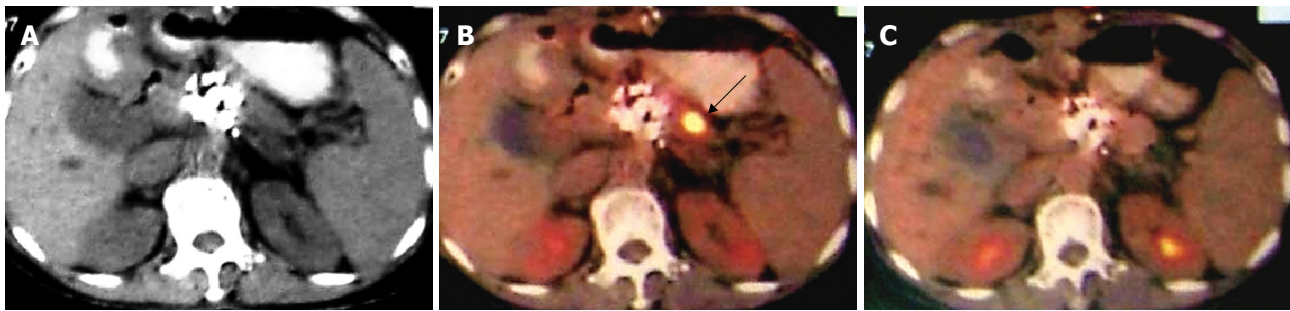


Figure 7 CT and PET-CT in case 6. **A**: CT at 10 mo after the first treatment, showing ^{125}I iodine seeds; **B**: PET-CT at 12 mo after the first treatment showing new lesion in pancreatic body; **C**: PET-CT at 3 mo after second treatment.

complete cryoablation to all the targeted tissue. Tumor persistence or recurrence at the site of cryoablation is often the result of incomplete destruction. Temperatures lower than -40°C are believed to be necessary for tumor ablation. Ice-balls targeted lesions are thus necessary for complete destruction of the tumor, because the outer several millimeters of the iceball circumference are at nonlethal temperatures. The 1-cm ice-ball extension beyond the tumor borders is required for adequate ablation^[19,20]. However, because the pancreatic volume is relatively small, cancer often involves most of the gland, and over-freezing increases the risk of complications, it is often difficult to ensure the “1 cm safe border”. Therefore, we decided to use the combination of cryosurgery with ^{125}I iodine seed implantation for the treatment of the pancreatic cancer. ^{125}I iodine with a half-life of 59 d provides γ radiation for a short distance, resulting in the death of the targeted cells. Brachytherapy using ^{125}I iodine seed implantation has been successfully used for the treatment of prostate cancer and metastatic or recurrent cancer^[21-24]. As a result, the use of ^{125}I iodine seed implantation is likely to be complementary to cryosurgery.

In the present study, 49 patients with locally advanced pancreatic cancer were treated with a combination of cryosurgery and ^{125}I iodine seed implantation. Thirteen patients underwent intraoperative cryosurgery and 36 patients percutaneous cryosurgery under ultrasound and CT guidance. The tumors showed different degrees of necrosis, and the CR, PR and SD were 20.4%, 38.8% and 30.6%, respectively, and only 10.2% demonstrated PD. During the median follow-up of 18 mo (5-40 mo), the median survival was 16.2 mo, of whom 26 patients (53.1%) survived 12 mo or more. The 6-, 12-, 24- and 36-mo survival rates were 94.9%, 63.1%, 22.8% and 9.5%, respectively.

Currently, the conventional therapies for locally

advanced pancreatic cancer are chemotherapy and radiotherapy. Previous reports showed a median survival of 6-10 mo in patients with locally advanced disease treated with 5-FU-based chemoradiation. Patients with metastatic disease had a shorter survival (3-6 mo)^[1]. A recently described combination regimen that is under investigation consists of gemcitabine, 5-FU, cisplatin, capecitabine and/or radiation^[25-34]. These combination therapies produced a median progression-free survival ranging from 3-10 mo, and median survival of 7-16 mo, the objective response rate of the tumors was 22%-40%, and 1-year survival was 20%-78% (less than 60% in most reports) (Table 3). The results in our series were similar to those reported previously. However, it is important to note that in this series there were 8 cases who survived for 24 mo or more. The patient with the longest survival is living for 40 mo, with no evidence of recurrence. The findings indicate that combination of cryosurgery and ^{125}I iodine seed implantation offers the possibility of complete remission.

Using univariate and multivariate analysis, presence of hepatic metastasis was an independent prognostic factor and was associated with poor outcome. It was surprising to note that patients who were underwent to adjuvant regional chemotherapy had a lower survival. This finding could in part be related to patient selection; patients receiving chemotherapy had more severe illness, and one-half had hepatic metastases.

By univariate analysis, it was observed that patients with cancer of pancreatic head had longer median survival compared with patients with cancer of pancreatic body or tail. The reasons may be that cancer of pancreatic head is detected relatively earlier because of the development of obstructive jaundice.

It is believed that tumor size is of critical importance in cryotherapy^[35]. However, tumor size could not be

Table 3 Recent chemoradiation trials in patients with locally advanced pancreatic cancer

Reporter	No. of patients (n)	Therapy	Median progression-free survival (mo)	Median survival (mo)	Objective response (%)	Survival at 12 mo after treatment (%)
El-Rayer ^[25]	47	Gemcitabine, cisplatin, and infusional fluorouracil				34
Tokuuye ^[26]	53	Small-field radiotherapy in combination with concomitant chemotherapy		10.2		35.2
Okusaka ^[27]	34	Gemcitabine + 5-FU	3.2	7.1	25	14.3
Yamazaki ^[28]	22	Concurrent chemoradiotherapy/gemcitabine		16	32	78
Isacoff ^[29]	50	5-FU, mitomycin dipyridamole			26	54
Park ^[30]	45	Gemcitabine + capecitabine	5.4	10.4	40	
Ko ^[31]	25	Gemcitabine + cisplatin, re-radiation + capecitabine	10.5	13.5		62
Polyzos ^[32]	32	Gemcitabine + 5-FU, folic acid, somatostatin	7	7	22	20
Michael ^[33]	30	Gemcitabine + 13-cis		7.8		
Furuse ^[34]		Intraoperative radiation, 5-FU infusion		7.8		8.1 (2 yr)
Present series	38	Cryosurgery and ¹²⁵ Iodine seed implantation		12	CR + PR 59.2	63.1

confirmed as an independent prognostic factor in our analysis. This finding may be related to the possibility that the combination of cryosurgery and ¹²⁵Iodine seed implantation may effectively destroy the entire tumor or a greater part of the targeted tissue, even in the presence of a large mass.

A great deal of attention has been paid to the safety of cryosurgery in the treatment of pancreatic cancer. Korpan^[8] performed an experimental study on dogs who received pancreatic cryosurgery using the disc cryoprobe. None of the animals developed complications and there was no cryosurgery-related mortality. Moreover, there was no post-cryosurgery bleeding, pancreatic fistulae or secondary infection. In our series, no cryosurgery-related mortality was observed. The main adverse effects were abdominal pain, fever and increased serum amylase levels. Some patients developed acute pancreatitis, but none had a poor outcome. In addition, ¹²⁵Iodine seed implantation can be performed at the same time, and is not accompanied with the adverse effects observed with chemo-radiotherapy. As a whole, combination therapy of cryosurgery and ¹²⁵Iodine seed implantation is a less invasive procedure.

Korpan^[8,18] pointed out that there were almost no known contraindications to the use of cryosurgery for pancreatic cancer. For most patients with pancreatic cancer, cryosurgery can substitute conventional surgery. These observations need to be confirmed by more studies. According to our experience, cryosurgery has several advantages in the treatment of unresectable pancreatic cancer: (1) The conventional management of unresectable pancreatic cancer involves a bypass operation without removal of the tumor. Cryosurgery can make up this shortcoming of conventional therapy, by converting the surgery from “palliative” to “radical”. (2) Cryosurgery is less invasive, and has lower rate of complications compared with conventional resection. (3) Unresectable tumors can be treated with percutaneous cryosurgery under ultrasound or CT guidance, with similar efficacy as intraoperative cryosurgery and is much less invasive to the patient. (4) During percutaneous cryosurgery, other modalities, such as ¹²⁵Iodine seed implantation, can be used simultaneously. (5) Metastatic tumors can be treated simultaneously, using the combination technique. (6)

Immune enhancement or activation after cryosurgery may occur probably due to quantitative and qualitative changes in the surface antigen (component) of tumor cells^[36]. That is called “cryoimmunity”^[37]. (7) The cryoablated cancerous tissue has increased sensitivity to chemo/radiotherapy^[38,39].

In conclusion, although the present data is preliminary, it indicates that combination of cryosurgery and ¹²⁵Iodine seed implantation may play an important role in the treatment of locally advanced pancreatic cancer. These findings warrant further refinement of the technique as well as initiation of controlled clinical studies to better define the true value of combination treatment in pancreatic cancer.

COMMENTS

Background

Pancreatic cancer is the fifth leading cause of cancer-related death for both men and women. Patient survival depends on the extent of the disease and patient's performance status at diagnosis. Patients who undergo surgical resection for localized non-metastatic pancreatic cancer have an approximately 20% longer survival rate, with a median survival of 12-20 mo. However, patients with locally advanced disease have a median survival of only 6-10 mo. The current approach of using chemoradiation, including gemcitabine, has failed to improve the outcome of this disease. Therefore, it is important to develop newer treatment modalities which are able to improve tumor control without the increasing toxicity in patients with locally advanced pancreatic cancer.

Research frontiers

Recently, cryosurgery has provided encouraging results in the treatment of prostate cancer and liver cancer. However, there is limited clinical experience using cryosurgery for the treatment of pancreatic cancer. Moreover, the use of ¹²⁵Iodine seed implantation has not been reported in the treatment of pancreatic cancer.

Innovations and breakthroughs

To our knowledge, this is the first report on the use of combined cryosurgery and ¹²⁵Iodine seed implantation in the treatment of locally advanced pancreatic cancer. Both cryosurgery and ¹²⁵Iodine seed implantation are local ablative techniques, with different mechanisms of action, and it is proposed that their combined use may have a complementary effect.

Applications

Cryosurgery and ¹²⁵Iodine seed implantation can be performed during surgery or percutaneously. Both techniques are mini-invasive modalities and can be adapted to treat unresectable tumor. In more than 80% of patients with pancreatic cancer,

surgical resection is not feasible at the time of diagnosis. Of the patients who undergo an operation with curative intent, only 30%-50% have successful removal of the tumor. Therefore, cryosurgery and ¹²⁵Iodine seed implantation are of special significance in the management of unresectable pancreatic cancer.

Terminology

Pancreatic cancer is derived mainly from ductal tissue with adenocarcinoma being the most common malignancy. There are very few pancreatic cancers which are classified as adenosquamous, giant cell cancers, and mucinous cystadenocarcinomas. Microscopically, these tumors may vary from well-differentiated to undifferentiated tumors. Seventy to 80 percent of respectable pancreatic cancers have already spread into lymph nodes at diagnosis. Ultrasonography and CT are the principal means of diagnosis of pancreatic cancer.

Peer review

This is an interesting and well written paper of much practical value. The presentation is adequate and easy to understand. The results of this paper, despite the limited case number and the short follow-up, suggest that benefit exists in the treatment of locally advanced pancreatic cancer with combined cryosurgery and ¹²⁵Iodine seed implantation.

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RAPID COMMUNICATION

Changes of histology and expression of MMP-2 and nm23-H1 in primary and metastatic gastric cancer

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Wang LB, Jiang ZN, Fan MY, Xu CY, Chen WJ, Shen JG. Changes of histology and expression of MMP-2 and nm23-H1 in primary and metastatic gastric cancer. *World J Gastroenterol* 2008; 14(10): 1612-1616 <http://www.wjgnet.com/1007-9327/14/1612.asp> <http://dx.doi.org/10.3748/wjg.14.1612>

Abstract

AIM: To investigate the changes of histology and expression of MMP-2 and nm23-H1 in primary and metastatic gastric cancer.

METHODS: One hundred and seventy-seven gastric cancer patients with lymph node and/or distal metastasis between 1997 and 2001 were reviewed. Differences in histology of the primary and metastatic gastric cancer were assessed. MMP-2 and nm23-H1 immunoreactivity was compared in 44 patients with tumor infiltration to the serosa layer.

RESULTS: Poorly and moderately differentiated metastatic gastric cancer was found in 88.7% (157/177) and primary gastric cancer in 75.7% (134/177) of the patients. The histological type of metastatic gastric cancer that was not completely in accordance with the preponderant histology of primary gastric cancer was observed in 25 patients (14.1%). MMP-2 immunoreactivity in metastatic gastric cancer was significantly stronger than that in primary gastric cancer, while nm23-H1 immunoreactivity showed no difference in primary and metastatic gastric cancer.

CONCLUSION: Metastatic gastric cancer presents more aggressive histological morphology and higher MMP-2 immunoreactivity than primary gastric cancer. This heterogeneity may elicit a possible mechanism of gastric cancer metastasis.

INTRODUCTION

Gastric cancer, one of the most common malignant diseases in the world, has been shown to frequently metastasize. Certain studies have reported the possible mechanisms underlying its metastasis^[1-3]. However, whether there is a histological difference between primary and metastatic gastric cancer is unclear and has been rarely reported.

Matrix metalloproteinases (MMPs) are defined as a family of enzymes which degrade extracellular membrane proteins, thus playing a significant role in tumor invasion and metastasis^[4]. MMP-2 is one of the most extensively studied MMPs in the process of cancer. It was reported that elevated MMP-2 level is related to increased tumor metastasis and stage in the lung, breast, stomach and colon^[5-8]. However, differences in MMP-2 expression between primary and metastatic gastric cancer have been rarely assessed.

The nm23 gene is a putative metastasis suppressor gene originally identified in metastatic murine melanoma cells^[9]. Reduction in nm23 expression is related with a high incidence of lymph node metastasis or poor prognosis of gastric cancer^[10,11]. However, there is no inverse relationship between nm23 expression and metastatic potential of gastric cancer^[12]. Their relationship in gastric cancer remains controversial.

Our study was to investigate the differences in histology and expression of MMP-2 and nm23-H1 between primary and metastatic gastric cancer, and to elucidate the possible mechanism of tumor heterogeneity underlying gastric cancer metastasis.

MATERIALS AND METHODS

Patients and tissue specimens

Complete data and tissue specimens were obtained from

230 gastric cancer patients, who underwent resection of gastric cancer at Sir Run Run Shaw Hospital, Zhejiang University College of Medicine between June 1997 and January 2001. Among them, 177 patients including 124 males and 53 females, ranging in age from 20 years to 79 years with a mean age of 55.5 years, had pathologically confirmed lymph node and/or distal metastasis and were enrolled to assess the differences in histology between primary and metastatic gastric cancer. Their clinicopathological features are shown in Table 1. Disease stage was classified based on the 5th edition of the International Union against Cancer and the American Joint Committee for Cancer Staging.

Forty-four patients, who had pathologically confirmed tumor infiltration to the serosal layer (24 pts, T₃N₁₋₃M₀, 20 pts, T₃N_xM₁), were recruited to evaluate the difference in MMP-2 and nm23-H1 immunoreactivity between primary and metastatic gastric cancer by immunohistochemistry.

Slides of tissue from primary and metastatic gastric cancer were observed by two pathologists. Following the criteria of World Health Organization (WHO), papillary adenocarcinoma was classified as well differentiated type, signet cell carcinoma and mucous adenocarcinoma as poorly differentiated type, tubular adenocarcinoma as well or moderately or poorly differentiated type.

We set the largest proportion of histological type of primary gastric cancer as the preponderant histological type. Thereby, percentage of the preponderant histological type of metastatic gastric cancer was as follows: -: lower than 5%; +: 5%-25%; ++: 25%-50%; +++: 50%-75%; ++++: higher than 75%. Comparison of changes in histology between primary and metastatic gastric cancer was made based on the percentages of their preponderant histological type.

Immunohistochemistry

Immunohistochemical study was performed using the following antibodies: anti-nm23-H1 protein (GE-213, monoclonal, 1:100; Manxin, Fuzhou, China) and anti-MMP-2 (CA-4001, monoclonal, 1:50; Manxin, Fuzhou, China). Four- μ m thick sections of 10% formalin-fixed, paraffin-embedded gastric cancer tissue were cut, mounted on glass slides coated with 3-aminopropyltriethoxysilane, and air-dried overnight at 60°C. The sections were deparaffinized in xylene and rehydrated in ethanol. Endogenous peroxidase was blocked with methanol containing 3% hydrogen peroxidase for 25 min. For staining with anti-MMP-2, sections were pretreated with citrate buffer (0.01 mol/L, pH 6.0) and heated at 100°C in a microwave oven for 20 min. For staining with anti-nm23-H1, sections were pretreated with trypsin (0.5%, pH 7.4) for 20 min at room temperature. The sections were incubated with primary antibodies at 4°C overnight, stained with a streptavidin-biotin-peroxidase kit (Manxin, Fuzhou, China), and reacted in a solution containing 3, 3'-diaminobenzidine and peroxyltrichloride substrate, and counterstained with hematoxylin. The provided sections known to react positively with nm23-H1 or MMP-2 (Manxin, Fuzhou, China) were used as a positive control. As a negative control, the primary antibody was deleted.

Table 1 Clinicopathologic data obtained from 177 gastric cancer patients

	Patients	
	<i>n</i>	%
Tumor size (cm) (mean \pm SD)	6.0 \pm 2.9	
Location		
Upper or whole body	40	22.6
Lower or middle body	137	77.4
Gross type		
Localized	39	22.0
Infiltrative	138	78.0
Depth of invasion		
T1	25	14.1
T2	86	48.6
T3	44	24.9
T4	22	12.4
Retrieved lymph nodes (mean \pm SD)	22.4 \pm 3.5	
Stage		
I	6	3.4
II	29	16.4
III	88	49.7
IV	54	30.5

Evaluation

The immunoreactivity of each antibody was evaluated. MMP-2 and nm23-H1 immunoreactivity was graded as -: without or with immunoreactivity in less than 5% tumor cells; +: immunoreactivity in 5%-25% tumor cells; ++: immunoreactivity in 25%-50% tumor cells; +++: immunoreactivity in over 50% of tumor cells.

Statistical analysis

All statistical analyses were conducted using the statistical program SPSS 10.0 for windows (SPSS, Chicago, IL, USA). Differences in histological morphology and expression of MMP-2 and nm23-H1 between each group were analyzed by chi-square test or by Fisher's exact test. $P < 0.05$ was considered statistically significant.

RESULTS

Histological changes

We observed different histological changes in primary and metastatic gastric cancer patients (Figure 1). Poorly and moderately differentiated metastatic gastric cancer was found in 88.7% (157/177) of the patients while primary gastric cancer in 75.7% (134/177) of the patients. The preponderant histological types of primary gastric cancer, graded as +++ and ++++, were more than those of metastatic lymph nodes (170 *vs* 138, $P < 0.01$). Moreover, the preponderant histological type of the metastatic lymph nodes in 14.1% patients (25/177) was not completely in accordance with that of primary gastric cancer (Table 2).

MMP-2 immunoreactivity

MMP-2 immunoreactivity was significantly stronger in metastatic gastric cancer than in primary gastric cancer. For the 20 patients with distal metastasis, a different MMP-2 immunoreactivity was observed in primary and metastatic gastric cancer (Table 3). MMP-2 immunoreactivity was stronger in metastatic gastric cancer than in primary gastric

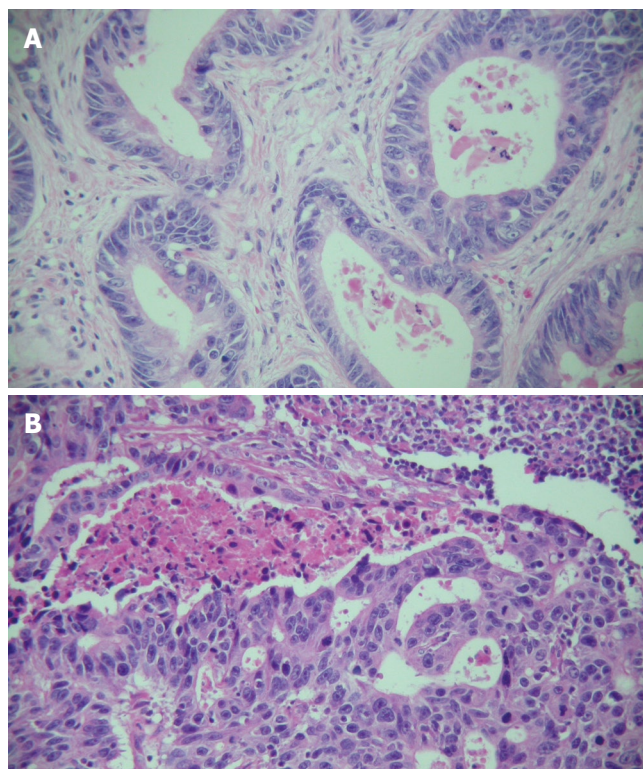


Figure 1 Histological changes in primary and metastatic gastric cancer (case 15) ($\times 200$). **A:** Histological change in primary gastric cancer showing a well differentiated adenocarcinoma of the stomach with a glandular pattern; **B:** Histological changes in metastatic gastric cancer showing a moderately differentiated adenocarcinoma with a cribriform pattern.

cancer. However, the immunoreactivity was similar in metastatic lymph nodes and distal metastasis (Figure 2).

nm23-H1 immunoreactivity

There was no significant difference in nm23-H1 immunoreactivity between primary and metastatic gastric cancer. The immunoreactivity was quite similar in primary and metastatic lymph nodes and distal metastasis (Table 4).

DISCUSSION

Studies on intratumoral and intertumoral heterogeneity have provided valuable insights into the pathogenesis and progression of different tumors^[13-15]. Although the concept of intratumoral heterogeneity of tumors has been generally accepted, studies on it in gastric cancer are scant. Previous reports focused mainly on comparison of molecular genetic alterations in each individual. However, this study investigated the tumor heterogeneity including histological morphology changes in primary and metastatic gastric cancer.

We observed different histological changes in primary and metastatic gastric cancer. Metastatic gastric cancer showed poorer differentiation. Meanwhile, the preponderant histological type of primary and metastatic gastric cancer was not completely identical. In the present study, the preponderant histological type of primary and metastatic gastric cancer site was different in 14.1% patients. These findings may imply that not all the histological types

Table 2 Preponderant histological type of primary and metastatic lymph nodes in 177 gastric cancer patients

Tumor type	Grade					P value
	-	+	++	+++	++++	
Primary lymph nodes	0	0	7	69	101	< 0.01
Metastatic lymph nodes	17	8	14	44	94	

Table 3 MMP-2 immunoreactivity in primary and metastatic T3 gastric cancer

Patients (n)	Tumor type	Grade				P value
		-	+	++	+++	
44 T ₃ N _x M _x Patients	Primary lesion	12	6	14	12	0.004
	Metastatic lymph node	7	6	9	22	
20 T ₃ N _x M ₁ Patients	Primary lesion	6	3	7	4	0.019
	Metastatic lymph node	4	3	4	9	
	Distal metastatic site	3	5	2	10	

Table 4 nm23-H1 immunoreactivity in primary and metastatic T3 gastric cancer

Patients (n)	Tumor type	Grade				P value
		-	+	++	+++	
44 T ₃ N _x M _x Patients	Primary lesion	12	15	15	2	0.138
	Metastatic lymph node	22	9	7	6	
20 T ₃ N _x M ₁ Patients	Primary lesion	8	4	7	1	0.497
	Metastatic lymph node	9	3	5	3	
	Distal metastatic site	10	3	5	2	

of primary gastric cancer have potential to metastasize, poorly differentiated cancer cells may play a significant role in lymph node metastasis, which may possibly explain why a small proportion of poorly differentiated cancer cells in primary gastric cancer may be preponderant in metastatic gastric cancer.

It is widely accepted that tumor may synchronously contain multiple histological types, reflecting different tumor differentiation and biological behavior. Most tumors may contain multiple cell clones with a diverse metastatic potential. Cell clones with a high metastatic potential are apt to metastasize to lymph nodes or distal organs^[16,17]. This dynamic heterogeneity may give a possible explanation for the different changes in histology between primary and metastatic gastric cancer. Although the preponderant histological change in primary gastric cancer is generally considered a prognostic predictor, the other histological changes in primary gastric cancer, especially in poorly differentiated subclones, may be as important as the preponderant histological change for the prognosis of primary gastric cancer. However, few studies have been addressed this issue, further investigations are warranted.

Because of its ability to degrade the basement membrane, MMP-2 has been postulated as a potential marker of tumor progression and prognosis in different malignancies such as ovarian cancer, gastric cancer and lung carcinoma^[8,18]. Schwartz *et al*^[19] reported that MMP-2 is expressed in SK-GT1, SK-GT5 and SK-GT6 but not in

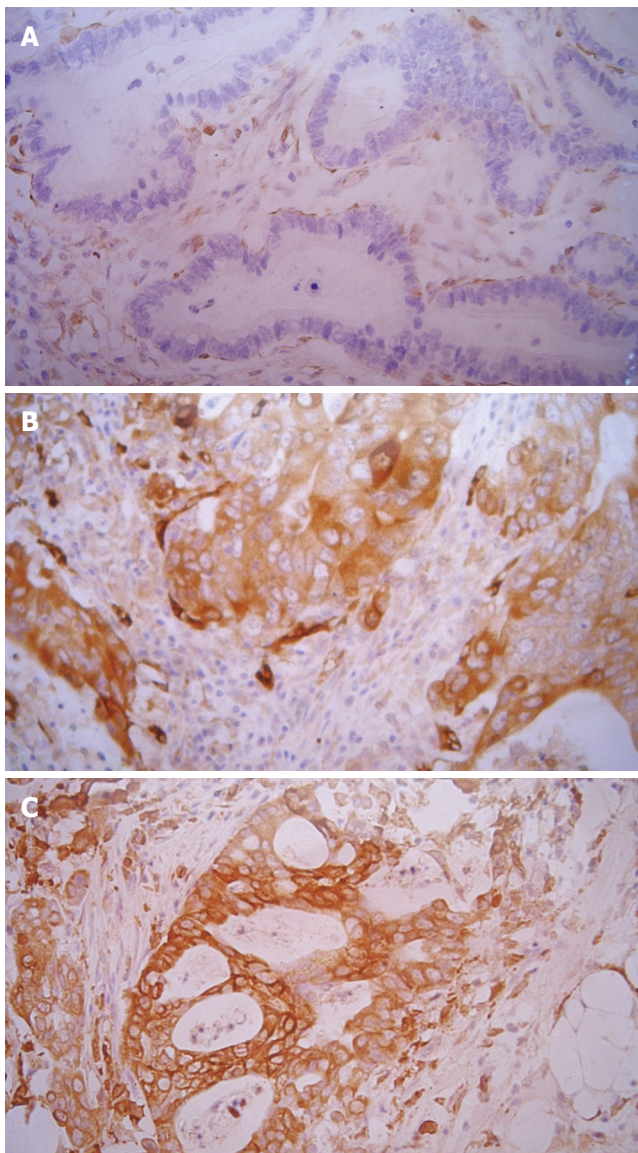


Figure 2 Immunohistochemistry of MMP-2 in gastric cancer (case 9) (× 200). **A:** Primary gastric cancer showing grade (-) immunoreactivity in MMP-2; **B:** Metastatic gastric cancer showing grade (+++) immunoreactivity; **C:** Distal metastatic gastric cancer showing grade (+++) immunoreactivity.

SK-GT2 and SK-GT4 gastric cancer cell lines. Ji *et al.*^[20] reported that MMP-2 expression is significantly higher in advanced than in early gastric cancer patients^[20]. These findings indicate that gastric cancer cells with a greater malignant and metastatic potential may secrete much more MMP-2 protein. Moreover, it has been shown that down-regulation of MMP or MMP-2 may inhibit tumor growth and metastasis, indicating that MMP-2 is correlated with gastric cancer invasion and metastasis^[21,22]. In this study, MMP-2 immunoreactivity was significantly higher in metastatic than in primary gastric cancer. This is in agreement with previous reports and suggests that when cancer tends to become invasive, elevated MMP-2 may play a pivotal role in its metastasis.

It was reported that Nm23 is a metastasis suppressor gene^[23]. However, its role in gastric cancer metastasis is controversial^[24,25]. Nakayama *et al.*^[26] demonstrated that reduced expression of nm23 is associated with gastric can-

cer metastasis. Similar results have been reported by Hsu *et al.*^[27]. However, Wang *et al.*^[28] found that patients with a high nm23 expression are easy to develop distal metastasis and have a lower nm23 expression, displaying that nm23 may play a trivial role in inhibiting tumor metastasis. Yeung *et al.*^[29] reported that there is no difference in nm23 expression between primary and metastatic gastric cancer. Similar results were observed in the present study, suggesting that nm23 expression is not associated with gastric cancer metastasis. Further study is needed to find the definitive role of nm23 in cancer metastasis.

In conclusion, metastatic gastric cancer is more aggressive and has a higher expression in tumor genes than primary gastric cancer. This heterogeneity may elicit one of the possible mechanisms underlying gastric cancer metastasis.

COMMENTS

Background

Gastric cancer metastasis occurs frequently and its possible mechanism has not been well addressed. Intratumoral and intertumoral heterogeneity plays a significant role in tumor progression. However, studies on gastric cancer are rarely reported.

Research frontiers

The aim of this study was to evaluate the morphology and tumor metastatic gene heterogeneity in gastric cancer.

Innovations and breakthroughs

This study showed the changes in morphology and expression of MMP-2 of primary and metastatic gastric cancer.

Applications

The heterogeneity in gastric cancer may provide a clue to the possible mechanism of cancer invasion and metastasis.

Peer review

This is a nice study comparing primary and metastatic gastric cancer. The expression of MMP2, one of the known metalloproteases relating to tumor invasion and metastases, was stronger in metastatic than in primary gastric cancer. However, NM23-H1 expression did not change in primary and metastatic gastric cancer. Primary and metastatic gastric cancer were found to have different histological types.

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Entecavir up-regulates dendritic cell function in patients with chronic hepatitis B

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CONCLUSION: Entecavir can enhance the biological activity of DCs derived from CHB patients.

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Key words: Chronic hepatitis B; Dendritic cell; Entecavir

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Lu GF, Tang FA, Zheng PY, Yang PC, Qi YM. Entecavir up-regulates dendritic cell function in patients with chronic hepatitis B. *World J Gastroenterol* 2008; 14(10): 1617-1621 Available from: URL: <http://www.wjgnet.com/1007-9327/14/1617.asp> DOI: <http://dx.doi.org/10.3748/wjg.14.1617>

Abstract

AIM: To investigate the *in vitro* effect of entecavir (ETV) on the function of dendritic cells (DCs) derived from chronic hepatitis B (CHB) patients.

METHODS: Mononuclear cells were isolated from peripheral blood of patients with CHB. DCs were incubated with RPMI-1640 medium supplemented with fetal bovine serum, IL-4, granulocyte-macrophage colony-stimulating factor (GM-CSF). DCs were treated with or without ETV on the fourth day. Cell surface molecules, including CD1a, CD80, CD83 and HLA-DR, were assessed by flow cytometry. Concentrations of IL-6 and IL-12 in the supernatant were assayed by enzyme-linked immunosorbent assay (ELISA). The ability of the generated DCs to stimulate lymphocyte proliferation was observed.

RESULTS: Compared with CHB control group, the expression levels of CD1a (29.07 ± 3.20 vs 26.85 ± 2.80), CD83 (25.66 ± 3.19 vs 23.21 ± 3.10), CD80 (28.00 ± 2.76 vs 25.75 ± 2.51) and HLA-DR (41.96 ± 3.81 vs 32.20 ± 3.04) in ETV-treated group were higher ($P < 0.05$). ETV-treated group secreted significantly more IL-12 (157.60 ± 26.85 pg/mL vs 132.60 ± 22.00 pg/mL ($P < 0.05$) and had a lower level of IL-6 in the culture supernatant (83.05 ± 13.88 pg/mL vs 93.60 ± 13.61 pg/mL, $P < 0.05$) than CHB control group. The ability of DCs to stimulate the proliferation of allogeneic lymphocytes was increased in ETV-treated group compared with CHB control group (1.53 ± 0.09 vs 1.42 ± 0.08 , $P < 0.05$).

INTRODUCTION

Hepatitis B virus (HBV) infection is a global public health problem, and over 400 million people suffer from HBV infection worldwide currently^[1,2]. Chronic HBV infection results from impaired antiviral immune response of the host that cannot produce sufficient competent specific cytotoxic T lymphocytes (CTL) to eliminate the invading virus^[3,4]. However, its mechanism remains unclear. Dysfunction of dendritic cells (DCs) is regarded as one of the factors for chronic hepatitis B (CHB) infection^[5]. DCs are crucial antigen-presenting cells responsible for initiating antiviral immune responses^[6,7]. Thus, one of the methods to treat CHB infection is to enhance the antigen presentation function of DCs in CHB patients, yet the precise mechanism needs to be further understood.

Entecavir (ETV), a nucleoside analogue, has been used in the clinical treatment of CHB infection because it can specifically inhibit the hepadnaviral DNA polymerase by competing with the corresponding dNTP for incorporation in ascent DNA and by acting on it as a chain terminator after incorporation^[8]. It appears to be transported into the cells *via* pyrimidine nucleoside transporters and is activated by several sets of cellular enzymes^[9]. Recent reports showed that lamivudine, a nucleoside analogue, can up-regulate the expression of major histocompatibility complex (MHC) class II^[10]. We hypothesize that ETV up-regulates DC function by increasing MHC and costimulatory molecules to enhance T lymphocyte immune response, thus strengthening the antiviral immune response. Therefore, we isolated DCs

from peripheral blood mononuclear cells of CHB patients, pulsed them with designated concentrations of ETV *in vitro* and observed its effects on DC phenotype and function. The results of this study provide new evidence to support the application of medicine and DC-based immunotherapy for CHB patients.

MATERIALS AND METHODS

Patients and materials

Twenty-five CHB patients with positivity HBsAg, HBeAg, HBcAb and serum HBV-DNA were enrolled in this study. All of them were negative for HCV and HIV and had no histories of other liver diseases. Ten healthy volunteers from postgraduates of Zhengzhou University were recruited into this study as controls (Table 1).

rhGM-CSF, rhIL-4, mouse anti-human HLA-DR-PE, CD80-FITC, CD1 α -FITC, CD83-PE were purchased from BioLegend, RPMI-1640 from GIBCO (USA), fetal calf serum (FCS) from Hangzhou Sijiqing Biological Engineering. Ficoll-Hypaque density gradient separate solution was purchased from Tianjin Jinmai Gene Biotechnology Company. rhIL-6 and IL-12 enzyme-linked immunosorbent assay (ELISA) kits (Peprotech) were purchased from Shanghai Shenxiong Technology Company. ETV was purchased from Bristol-Myers Squibb Company in Shanghai.

Preparation of DCs

Peripheral blood was collected from CHB patients and healthy volunteers and heparinized. Peripheral blood mononuclear cells (PBMC) were isolated by centrifuging on a column of Ficoll-Conray *in vitro* as previously described^[11-13]. Briefly, PBMC were suspended in RPMI 1640 medium supplemented 10% fetal bovine serum (FBS) and seeded in 24-well plastic plates for 2 h. The non-adherent cells were gently removed and the adherent cells were cultured in RPMI-1640 medium supplemented with 10% FBS, 10 ng/mL rhGM-CSF, 5 ng/mL rhIL-4 in a humidified atmosphere containing 50 mL/L CO₂ at 37°C. On the fifth day DCs from CHB patients were treated with or without ETV (0.05 μ g/mL) and designated as ETV treatment group and CHB control group, respectively. DCs from healthy volunteers were designated as healthy control group not treated with ETV. Half of the medium was replaced with a fresh medium every other day. DCs were harvested on the eighth day.

Morphological analysis and flow cytometry

DCs were observed under an inverted microscope. Surface makers of DCs, such as CD1a, CD80, CD83, HLA-DR, were analyzed by flow cytometry (FCM) on the eighth day using conjugated monoclonal mouse-anti-human antibodies (FITC-anti-CD1a, FITC-anti-CD80, PE-anti-CD83, PE-anti-HLA DR) as previously described^[14].

Allogeneic mixed leukocyte reaction (All MLR)

Mononuclear cells were isolated from peripheral blood of healthy subjects. After incubated for 2 h, the non-adherent cells were collected as lymphocytes. Mitomycin C (50 μ g/mL)

was added to the culture. DCs were harvested from each group after 30 min and then seeded onto 96-well culture plates (1×10^4 /well) as stimulator cells together with lymphocytes (1×10^5 /well) as responder cells. Triple wells were set for each group. After cultured for 72 h, OD570 was assayed by MTT and stimulator index (SI) was calculated following the formula of $SI = OD_{\text{experiment}} / (OD_{\text{responder cells}} + OD_{\text{stimulator cells}})$.

Measurement of IL-12 and IL-6 levels in culture supernatant of DCs

Concentrations of IL-12, IL-6 in the supernatant of DCs on the eighth day were detected with ELISA kits according to the manufacturer's instructions. Triple wells were set for each sample.

Statistical analysis

Data were analyzed with SPSS10.0 statistical software. The significant difference between groups was determined by one-way ANOVA. $P < 0.05$ was considered statistically significant.

RESULTS

Morphology analysis

After cultured for 24 h, swarming of cells was observed under a microscope. Two days later the size of DCs became larger and five days later much ecphyma was found on the surface of DCs, many nebulous substances floating on the culture liquid were demonstrated on the seventh day. However, the ETV-treated group had a distinct modal difference compared with the CHB control group (Figure 1).

Phenotype of DCs

Markers of DCs were examined by flow cytometry on the eighth day. Compared with healthy control group, The expression levels of CD1a, CD80, CD83 and HLA-DR on DCs were lower in the CHB control group than in the healthy control group (26.85 ± 2.80 vs 39.41 ± 3.12 , $P < 0.001$; 25.75 ± 2.51 vs 38.52 ± 3.18 , $P < 0.001$; 23.21 ± 3.10 vs 40.76 ± 3.15 , $P < 0.001$; and 32.20 ± 3.04 vs 59.62 ± 4.73 , $P < 0.001$), and were higher in the ETV-treated group than in the CHB control group (29.07 ± 3.20 vs 26.85 ± 2.80 , $P = 0.043$; 28.00 ± 2.76 vs 25.75 ± 2.51 , $P = 0.046$; 25.66 ± 3.19 vs 23.21 ± 3.10 , $P = 0.027$; and 41.96 ± 3.81 vs 32.20 ± 3.04 , $P < 0.001$) (Table 2).

Concentration of IL-12 and IL-6 in supernatant of DCs

The concentration of IL-12 was reduced more significantly in the CHB control group than in the healthy control group (132.60 ± 22.00 pg/mL vs 301.90 ± 39.54 pg/mL, $P < 0.001$), while the concentration of IL-6 was increased more significantly in the CHB control group than in the healthy control group (93.60 ± 13.61 pg/mL vs 44.10 ± 9.69 pg/mL, $P < 0.001$). DCs treated with ETV secreted more IL-12 than DCs in the CHB control group (157.60 ± 26.85 pg/mL vs 132.60 ± 22.00 pg/mL, $P = 0.041$) and had a lower level of IL-6 in the culture supernatant (83.05 ± 13.88 pg/mL vs 93.60 ± 13.61 pg/mL, $P = 0.042$, Figure 2).

Table 1 Clinical and serological data from patients studied (mean \pm SD)

Group	n	Age (yr)	HBsAg	HBeAg	Anti-HBc	HBV-DNA (IU/mL)	ALT ¹ (nkat/L)
Patients	25	25.7 (18-42)	+	+	+	> 2.0E + 04	2645.09 \pm 1799.87
Volunteers	10	26.2 (24-32)	-	-	-	NT	311.28 \pm 83.40

NT: not tested; ¹Normal range < 666.8 nkat/L.

Table 2 Expression of costimulatory molecules on DCs from different groups of patients (mean \pm SD)

	n	CD1a	CD80	CD83	HLA-DR
Healthy control DC	10	39.41 \pm 3.12	38.52 \pm 3.18	40.76 \pm 3.15	59.62 \pm 4.73
CHB control DC	25	26.85 \pm 2.80 ^b	25.75 \pm 2.51 ^b	23.21 \pm 3.10 ^b	32.20 \pm 3.04 ^b
ETV-treated DC	25	29.07 \pm 3.20 ^a	28.00 \pm 2.76 ^a	25.66 \pm 3.19 ^a	41.96 \pm 3.81 ^a

^a $P > 0.05$ vs CHB control group; ^b $P < 0.01$ vs healthy control group.

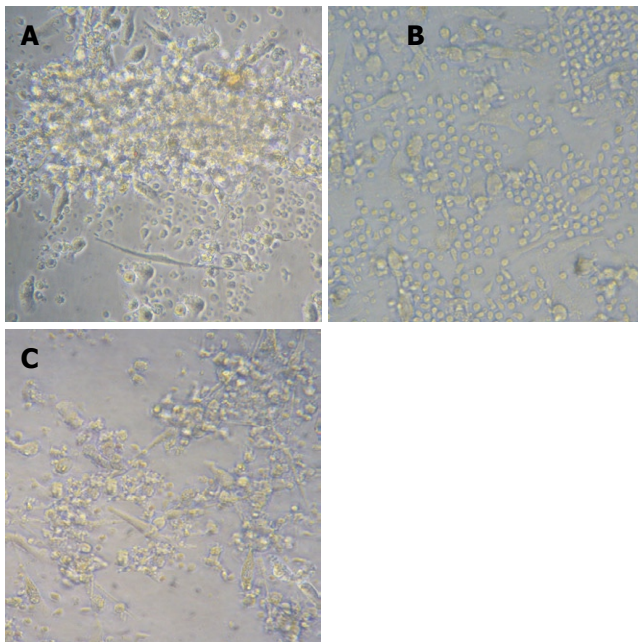


Figure 1 Morphology of DCs on d 8 (\times 400) in healthy control group (A), CHB control group (B), and ETV treatment group (C).

Priming lymphocytes *in vitro* pulsed with ETV-treated DCs

The stimulator index of DCs in the ETV-treatment group was markedly higher than that in the CHB control group (1.53 ± 0.09 vs 1.42 ± 0.08 , $P = 0.032$), and lower than that in the healthy control group (1.53 ± 0.09 vs 1.78 ± 0.09 , $P = 0.040$).

DISCUSSION

Weak and oligospecific antiviral B and T cell responses are responsible for the insufficient control of chronic HBV infection^[15]. However, the mechanism underlying this immunological hypo-responsiveness remains unknown^[16,17]. Immune tolerance may play an important role^[18,19]. It was reported that the immune system and cytokine play a key role in HBV clearance^[11]. Patients infected with HBV

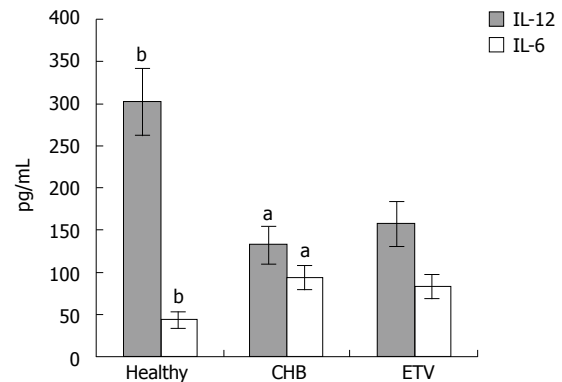


Figure 2 Secretion of IL-12 and IL-6 from different groups of patients (^a $P < 0.05$ vs ETV; ^b $P < 0.01$ vs CHB control).

start up a series of non-specific immunology responses including activation of natural killer cells and interferon. However, the complete clearance of HBV must rely on the activation of HBV-specific T lymphocytes^[20]. If HBV cannot be completely eliminated, the number of HBV-antigen specific CD8⁺ T lymphocytes would decrease, resulting in immune tolerance to the invading virus and CHB^[21].

DCs are antigen-presenting cells that link innate immunity with adaptive immunity and are essentially involved both in the initiation of primary immune responses and in the establishment of peripheral tolerance^[22]. The mechanism of CHB is related to the impairment of immune capacity resulting from functional deficiency of DCs in quality and quantity^[23]. HBV might cause phenotypic and functional alterations by directly affecting the DC precursors in blood or bone marrow^[14]. So one of the important pathways is to selectively modify DCs and activate HBV specific immune response during CHB treatment^[24]. Beckebaum *et al*^[10] argued that lamivudine can increase the expression of HLA-DR on DCs while processing HBV. Based on this fact, we cultured DCs for four days and pulsed them *in vitro* with entecavir, a new nucleoside analogue, at certain concentrations, and then observed the effect of ETV on DC phenotype and function.

The key mechanism underlying DC-mediated T cell stimulation after antigen uptake and process by APC includes engagement of the antigen-specific T cell receptor by peptide-loaded MHC molecules, interaction of costimulatory molecules with their receptors on T cells and secretion of cytokines^[25]. CD1a is a specific marker of human DCs, CD83 is the mature sign of human DC, and HLA-DR, one of the MHC II molecules, mostly takes part in the antigen presentation, while CD80, one of the co-stimulating molecules, promotes T cell activation by combining with the correspondent T cell receptor^[26,27]. A significantly reduced expression of CD1a, CD80, CD83 and HLA-DR on DCs from CHB patients was detected in the present study. Flow cytometry showed that the expression of cellular surface markers such as CD1a, CD80, CD83 and HLA-DR on DCs increased more significantly in the ETV treatment group than in the CHB control group. The enhanced stimulatory capacity of ETV-treated DCs in MLR indicates that other mechanisms may be involved in the activation and proliferation of T cells apart from the mechanism underlying the traditional double-signs.

DCs dictate Th0 cells to differentiate towards Th1 and Th2 cells accompanying secretion of IL-6, IL-12 and IFN- γ ^[28]. IL-12 secreted by mature DCs drives Th0 cells to develop into Th1 cells, promotes secretion of IL-2, IFN- γ and participates in the cellular immune response. IL-6 secreted by immature DCs is related to Th2 cell development, restrains the cellular immune response and induces immune tolerance^[29]. Since IL-12 plays a critical role in the Th1 cell differentiation, decreased IL-12 levels in CHB patients can be the factor that directly attributes to the weak T-cell stimulatory capacity of DCs from CHB patients in MLR^[30]. During our experiments, secretion of IL-12 increased more significantly in the ETV treatment group than in the CHB control group. The level of IL-6 was lower in the ETV treatment group than in the control group. Thus, we may conclude that DCs pulsed by ETV at designated concentrations can promote Th1 cell proliferation and enhance cellular immune response.

In conclusion, ETV could increase the expression of CD1a, CD80, CD83 and HLA-DR and the secretion of IL-12, reduce the secretion of IL-6 and enhance the proliferation of T cells in the present study, indicating that ETV can change the biological activities of DCs derived from CHB patients, which can be utilized as a DC-based immunotherapy for CHB infection. Finally, our data, similar to those of previous reports^[31], have a limitation in interpretation for immune pathology of chronic hepatitis B virus infection since the *in vitro* generated DCs can be found at different developing stages with variable functional aspects, although this study model has been employed in similar studies^[31].

COMMENTS

Background

Hepatitis B virus (HBV) infection is a global public health problem. Immune response of the host plays an important role in the pathogenesis of chronic HBV infection.

Research frontiers

One of the important factors responsive for the immune tolerance in chronic

hepatitis B (CHB) is the impaired function of dendritic cells (DCs) which cannot efficiently present HBV antigens to the host immune system.

Innovations and breakthroughs

DC-based therapeutic vaccine has recently been considered a potential approach to the treatment of CHB. Entecavir, a nucleoside analogue, specifically inhibits the hepadnaviral DNA polymerase and up-regulates the host immune system.

Applications

Results of the present study support the application of entecavir and DC-based immunotherapy for CHB.

Peer review

This is a good paper, describing that entecavir combined with DCs may be an effective therapeutic approach to eradication of chronic HBV infection.

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CASE REPORT

Pseudocirrhosis in a pancreatic cancer patient with liver metastases: A case report of complete resolution of pseudocirrhosis with an early recognition and management

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early recognition and management can lead to a near complete recovery of liver function and much improved quality of life as illustrated in this case.

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Key words: Pseudocirrhosis; Pancreatic cancer; Nodular regenerative hyperplasia; Chemotherapy induced liver toxicity

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Abstract

We report a case of pseudocirrhosis arising in the setting of regression of liver metastases from pancreatic cancer. A 55-year-old asymptomatic woman presented to our clinic with newly diagnosed metastatic pancreatic cancer with extensive liver metastases. She underwent systemic chemotherapy with gemcitabine and oxaliplatin (GEMOX). After 8 cycles of therapy, she had a remarkable response to the therapy evidenced by decline of carcinoembryonic antigen (CEA) and CA19 by > 50% and nearly complete resolution of hepatic metastases in computed tomography (CT) scan. Shortly after, she developed increasing bilateral ankle edema and ascites, associated with dyspnea, progressive weight gain, and declining performance status. Gemcitabine and oxaliplatin were discontinued as other causes of her symptoms such as congestive heart disease or venous thrombosis were ruled out. CT scan 6 mo after the initiation of GEMOX revealed worsening ascites with a stable pancreatic mass. However, it also revealed a lobular hepatic contour, segmental atrophy, and capsular retraction mimicking the appearance of cirrhosis. She was managed with aggressive diuresis and albumin infusions which eventually resulted in a resolution of the above-mentioned symptoms as well as complete resolution of pseudocirrhotic appearance of the liver and ascites in CT scan. This case demonstrates that pancreatic cancer patients can develop pseudocirrhosis. Clinicians and radiologist should be well aware of this entity as

INTRODUCTION

In patients with metastatic cancer involving the liver, treatment with chemotherapy can result in areas of retracted tumor tissue and scarring. This entity is referred to as pseudocirrhosis because it resembles macronodular cirrhosis radiographically and has been associated with hepatic decompensation. Pseudocirrhosis has been reported almost exclusively in patients undergoing treatment for metastatic breast cancer. We report a patient with pancreatic cancer and liver metastasis who developed pseudocirrhosis after achieving a clinical and radiographic response to gemcitabine and oxaliplatin.

CASE REPORT

A 55-year-old woman presented to our institution in November 2006 with newly diagnosed, untreated metastatic pancreatic cancer, as well as biopsy-proven liver metastases and peritoneal deposits. She was asymptomatic with an ECOG performance status of 0, had no risk factors for hepatitis or cirrhosis, and her physical examination was unremarkable. Her hemoglobin was 102 (120-160 g/L), albumin was 33 (35-50 g/L), alkaline phosphatase was 300 (30-130 U/L), and total bilirubin

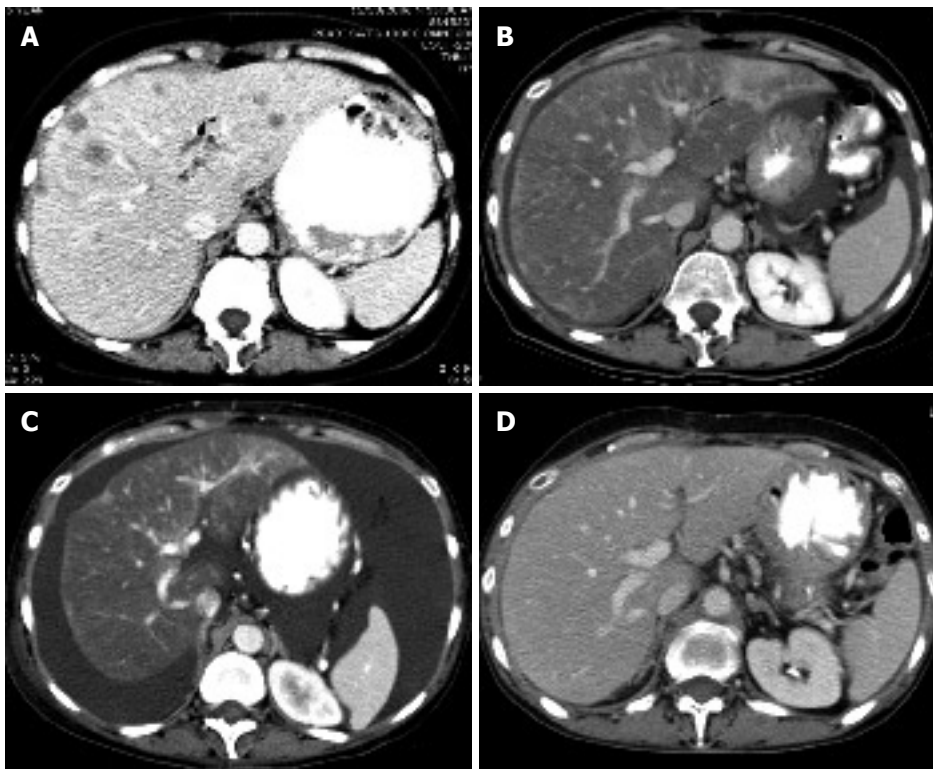


Figure 1 Computed tomography (CT) of the liver. CT scan is the initial imaging study. **A:** CT of the liver after contrast enhancement showing numerous liver metastases; **B:** CT scan 4 mo after initial study showing marked diminution of the metastases and marked fatty infiltration of the liver; **C:** The liver 2 mo after B showing no evidence of metastasis but findings which simulate cirrhosis with ascites, and irregular contours with retraction. This constellation of CT findings is consistent with a diagnosis of pseudocirrhosis; **D:** CT scan 2 mo after C showing a nearly normal liver and only a trace of ascites present in the pelvis (image not shown).

was 1.54 g/L (less than 1.20 g/L). CA19-9 and CEA were elevated at presentation to 464 (0-37.0 U/mL) and 12.5 (0-3.0 ng/mL), respectively. A CT scan of the chest, abdomen and pelvis in December 2006 revealed multiple bilateral lung nodules which measured 5 mm or less, a 4.7 cm × 3.2 cm neoplasm at the head of the pancreas, and innumerable liver metastases (Figure 1A).

The patient began systemic chemotherapy with gemcitabine (1000 mg/m²) and oxaliplatin (100 mg/m²) every other week in December 2006. On a restaging CAT scan obtained after 3 cycles of treatment, innumerable hepatic metastases appeared less conspicuous. After 8 cycles of the therapy, she remained clinically well, CEA and CA19 were declined by > 50%, and CT scan on 4/10/2007 revealed nearly complete resolution of pulmonary nodules and hepatic metastases while the pancreatic mass remained stable. Fatty infiltration of the liver and new ascites were noted (Figure 1B).

In May 2007, she developed increasing bilateral ankle edema and ascites, associated with dyspnea, progressive weight gain, and declining performance status. Gemcitabine and oxaliplatin were discontinued. The ascites and fluid retention continued to worsen despite escalation of diuretics, and serum albumin decreased to 24 g/L, cardiac echocardiogram and duplex Doppler ultrasound of the lower extremities revealed no evidence of congestive heart disease or venous thrombosis. CT scan on June 7, 2007 revealed worsening ascites with a stable pancreatic mass. However, it also revealed a lobular hepatic contour, segmental atrophy, and capsular retraction mimicking the appearance of cirrhosis (Figure 1C). Hepatic Doppler ultrasound on June 21, 2007 revealed patent splenic, portal confluence, and hepatic veins with normal direction of flow. Her albumin declined to a nadir of 17 g/L. Bilirubin

and ALT (SGPT) were normal, and AST (SGOT) was < 2X upper limit of normal. The patient underwent a large volume paracentesis and the serum-to-ascites albumin gradient was greater than 11 g/L, indicating portal hypertension as the cause of her ascites. She was managed with aggressive diuresis and weekly albumin infusions. Over the next three months, she had marked improvement in her overall status with resolution of peripheral edema, diminished ascites, and normalization of albumin to 37 g/L. The patient's follow-up CT scan 14 wk after discontinuation of chemotherapy revealed a near resolution of pseudocirrhotic appearance of the liver and ascites along with a decrease in the size of the pancreatic head mass (Figure 1D). CA19-9 was decreased further to 64.

DISCUSSION

In patients with metastatic cancer involving the liver, treatment with chemotherapy can result in areas of retracted tumor tissue and scarring. This entity is referred to as pseudocirrhosis because it resembles macronodular cirrhosis radiographically and can be associated with hepatic decompensation, while lacking of the classic pathologic attributes of cirrhosis^[1]. Pseudocirrhosis has been reported almost exclusively in patients undergoing treatment for metastatic breast cancer. We report herein the first case of pseudocirrhosis arising in a patient with metastatic pancreatic cancer.

A wide range of chemotherapeutic agents are associated with pseudocirrhosis in patients with breast cancer, including adriamycin, cyclophosphamide, 5-fluorouracil, methotrexate, cisplatin, carmustine, tamoxifen, paclitaxel, megestrol acetate, vinblastine, etoposide, thiotepa, ifosfamide, navelbine,

and vincristine^[1-3]. Patients exhibit radiographic findings of cirrhosis, such as capsular retraction with volume loss and lobulation of the liver contour adjacent to the treated metastatic disease^[1,3-5]. The degree of change tends to correlate with the extent of metastatic burden in the liver^[4]. In the series by Young *et al*, 52% and 27% of cases resulted in ascites and splenomegaly, respectively^[1]. While it is rare, there are case reports on severe manifestations of portal hypertension, such as hepatic encephalopathy or variceal bleeding, associated with pseudocirrhosis, suggesting that the life threatening clinical consequences can be equivalent to those seen in classic cirrhosis despite the differences in respective pathophysiology^[1,3,6].

The mechanism of pseudocirrhosis arising in the setting of chemotherapy-induced regression of liver metastases is unclear. It has been proposed that tumor shrinkage and subsequent scar formation around the treated liver lesions are a possible mechanism, since volume loss and capsular retraction typically occur adjacent to the treated metastases. Alternatively, nodular regenerative hyperplasia (NRH) in response to chemotherapy-induced hepatic injury has been proposed as the causal mechanism of pseudocirrhosis^[1,7,8]. The formation of regenerative hepatic nodules with subsequent compression and atrophy of intervening parenchyma without hepatic fibrosis is the hallmark of NRH^[1]. NRH has been associated with various chemotherapeutic agents including oxaliplatin, 5-FU, and agents used in acute leukemia^[7,9-11]. Oxaliplatin has been linked to the development of hepatic sinusoidal lesions, with areas of hepatic regeneration which can occasionally reach a pattern of NRH. However, clinically significant hepatic decompensation in association with these histopathologic findings has not been reported with oxaliplatin. Gemcitabine-associated liver toxicities have been very rarely reported^[12-15].

To our knowledge, this represents the first reported case of pseudocirrhosis arising in the setting of regression of liver metastases from pancreatic cancer. The absence of a liver biopsy is the major limitation in fully understanding the etiology of this patient's pseudocirrhosis. Since this patient had dramatic resolution of liver metastases and declining tumor markers prior to developing pseudocirrhosis, we cannot distinguish between tumor regression with scar formation or hepatotoxic effects of chemotherapy as potential mechanisms. However, the complete resolution of the radiographic and clinical hallmarks of pseudocirrhosis with cessation of chemotherapy suggests that, in this case, pseudocirrhosis was due, in part, to chemotherapy-associated hepatic injury.

In conclusion, this case illustrates that pseudocirrhosis can occur in pancreatic cancer as well as in breast cancer. Clinicians should be aware of this entity when treating patients with extensive liver metastases from pancreatic cancer. Early recognition and appropriate management, including discontinuation of implicated chemotherapeutic agents, cannot only prevent further liver damage and life-

threatening consequences of portal hypertension, but can also lead to a full recovery of liver function.

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A case of asymptomatic intraductal papillary neoplasm of the bile duct without hepatolithiasis

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findings and emphasize that cholangiography is especially helpful for the diagnosis of bile duct dilatation due to infiltration of carcinoma cells.

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Key words: Cholangiocarcinoma; Intraductal papillary neoplasm; Dilated bile duct; Hepatectomy; MUC

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Hayashi J, Matsuoka S, Inami M, Ohshiro S, Ishigami A, Fujikawa H, Miyagawa M, Mimatsu K, Kuboi Y, Kanou H, Oida T, Moriyama M. A case of asymptomatic intraductal papillary neoplasm of the bile duct without hepatolithiasis. *World J Gastroenterol* 2008; 14(10): 1625-1629 Available from: URL: <http://www.wjgnet.com/1007-9327/14/1625.asp> DOI: <http://dx.doi.org/10.3748/wjg.14.1625>

Abstract

A 65-year-old woman was found to have dilatation of the intrahepatic bile duct in the right anterior segment during a general health. Laboratory data were within normal ranges and no solid mass was detected in her abdominal computer tomography (CT) or nuclear magnetic resonance imaging (MRI). However, endoscopic retrograde cholangiopancreatography (ERCP) demonstrated an obstruction of the right bile duct. Intraoperative cholangiography showed stenosis of the intrahepatic bile duct in the anterior inferior segment (B5) and narrowness of the intrahepatic bile duct in the anterior superior segment (B8), so that we strongly suspected intrahepatic cholangiocarcinoma (ICC). Histologically, surgically resected liver specimens, without tumor mass by macroscopic observation, showed intraductal papillary proliferation with fibrovascular cores and intraductal spreading of carcinoma *in situ* throughout a considerable area, especially in bile ductules around the peripheral small portal area. Furthermore, the immunohistochemical profile of the tumor (MUC5AC+/CK7+) was compatible with an intraductal papillary neoplasm of the bile duct (IPN-B). Consequently, this case was diagnosed as IPN-B with spreading CIS, stage I (pT1, pN0, P0, H1, M0). We report a case of IPN-B with interesting histopathological

INTRODUCTION

Intraductal papillary neoplasm of the bile duct (IPN-B) is a neoplastic lesion preceding invasive intrahepatic cholangiocarcinoma (ICC) and is a new definition of a tumor with papillary growth in the intra- or extra-hepatic bile duct^[1,2]. Histologically, IPN-B is characterized by a prominent papillary growth of atypical biliary epithelium with distinct fibrovascular cores and, frequently, mucin-secretion. It has previously been described as biliary papillomatosis^[3], bile duct cystadenocarcinoma^[4], intrahepatic cholangiocarcinoma (ICC)^[5] or a mucin hypersecreting bile duct tumor^[6].

On the other hand, biliary intraepithelial neoplasia (BilIN) showing, microscopically, growth of atypical biliary epithelium, has been identified as another type of neoplastic lesion preceding ICC^[7]. BilIN is known often to progress to tubular adenocarcinoma, while IPN-B is associated with mucinous carcinoma and tubular adenocarcinoma^[1,2,8]. Biliary papillary tumors, including IPN-B, resemble, histologically, intraductal papillary mucinous neoplasms of the pancreas (IPMN-P)^[9,10]. In both organs, these neoplasms arise within the duct system and show a predominantly intraductal growth pattern, commonly an overproduction of mucin and an association with invasive adenocarcinoma^[11].

Based on gross morphology, ICC can be divided into three types: mass-forming, periductal-infiltrating and intraductal growth types^[12]. Of these, the intraductal growth type, which corresponds to IPN-B, is associated with the most favorable outcome^[13]. One feature of IPN-B is its relatively good prognosis after complete hepatic resection^[14,15]. Therefore, it is important to make a precise diagnosis at an early stage and to perform early surgical resection.

We report the clinical and histological findings of a patient who was diagnosed as IPN-B without hepatolithiasis and underwent a hemihepatectomy.

CASE REPORT

A 65-year-old woman was found to have dilatation of the intrahepatic bile duct in the right anterior segment during a general health examination in our hospital (Figure 1A). She had no history of liver disease, including hepatolithiasis. She was admitted to our hospital for detailed examination. Physical examination on admission revealed a height of 152.3 cm, a weight of 45 kg, a temperature of 37.1°C, a blood pressure of 117/61 mmHg, a pulse rate of 78/min, and a respiration rate of 22/min. Pertinent laboratory values included a white blood cell count (WBC) of 3180/ μ L, aspartate aminotransferase (AST) level of 30 IU/L, alanine aminotransferase (ALT) level of 30 IU/L, alkaline phosphatase (ALP) level of 400 IU/L, γ -glutamyl transferase (γ -GTP) level of 15 IU/L, CEA of 1.4 ng/mL, CA19-9 of 3 U/mL, and AFP of 5.6 ng/mL. Abdominal computed tomography (CT, Figure 1B) and magnetic resonance imaging (MRI) showed dilatation of the intrahepatic bile duct in the right anterior segment, but no solid mass around it. No metastases were found inside or outside the liver. Celiac angiography did not reveal any hypervascular tumors. Endoscopic retrograde cholangiopancreatography (ERCP) demonstrated an obstruction of the right bile duct at the root of the right hepatic duct but the common and left intrahepatic bile ducts were not dilated (Figure 2). These findings suggested that the dilatation of bile duct was due to the invasion of ICC and prompted us to plan a curative excision.

Neither ascites nor a palpable tumor of the liver was detected during laparotomy. Intraoperative US showed only dilatation of the bile duct (B5), as had been detected preoperatively. Subsequently, cholangiography demonstrated stenosis of the B5 bile duct and narrowness of the B8 bile duct (Figure 3). This feature was strikingly suspicious of cholangiocarcinoma, so we elected to perform a right-lobule hepatectomy. An intraoperative frozen section was negative for malignancy in the margin of the right bile duct.

Macroscopic examination of the resected liver revealed bile duct dilatation in the right hepatic lobe (Figure 4). However, we could not identify any mass lesions and mucin was not observed macroscopically.

Microscopically, intrahepatic bile ducts were dilated with slight peri-ductal fibrosis. Within the dilated bile ducts, atypical biliary epithelium proliferated in a papillary fashion, associated with fine fibro-vascular cores (Figure 5A). Atypical cells showed nuclear enlargement, irregular nuclear

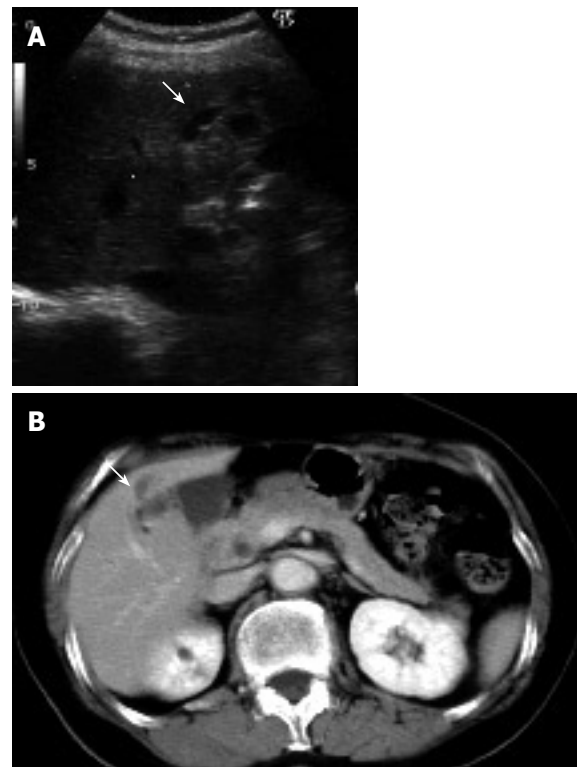


Figure 1 A: Abdominal ultrasonography (US) of the liver showing slight dilatation of bile ducts in the right liver (S5); B: Contrast-enhanced CT scan of the abdomen showing markedly dilatation of bile ducts in the liver (S5). Solid masses were not observed.



Figure 2 Endoscopic retrograde cholangiopancreatography (ERCP) showing a biliary obstruction at the root of the hepatic duct, suggesting cholangiocarcinoma.

membranes and distorted cellular polarity (Figure 5B). These atypical features corresponded to carcinoma *in situ*. Carcinoma cells proliferated continuously from the intrahepatic large bile ducts to small bile ducts (Figure 5C). Interestingly, carcinoma cells also were observed in proliferating bile ductules, which showed an irregular arrangement and slight dilatation (Figure 5D). Portal tracts showing irregular bile duct dilatation resembled Caroli's disease; however, we could not identify any features of Caroli's disease in the non-neoplastic area. No invasive growth could be detected.

Immunohistochemical examination revealed that tumor cells were positive for CK7, CK20 and MUC5AC, and negative for MUC1, MUC2, and p53 (Figure 6). Finally, we diagnosed this lesion as IPN-B (carcinoma *in situ*), showing



Figure 3 Cholangiography showing stricture of the bile duct (B5) and narrowness of bile duct (B8) in the right liver.

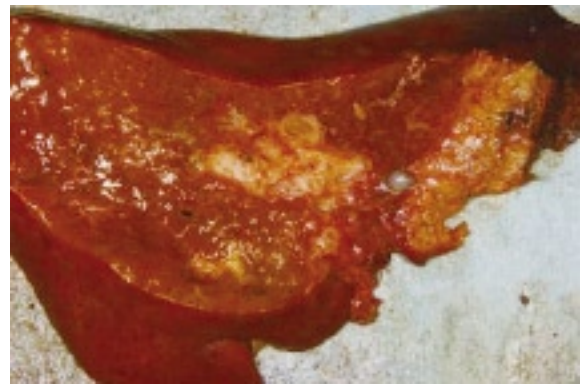


Figure 4 Macroscopic appearance of the resected right liver showing dilatation in the stump of the right intrahepatic duct, without a solid tumor component, which is surrounded by a fibrotic area.

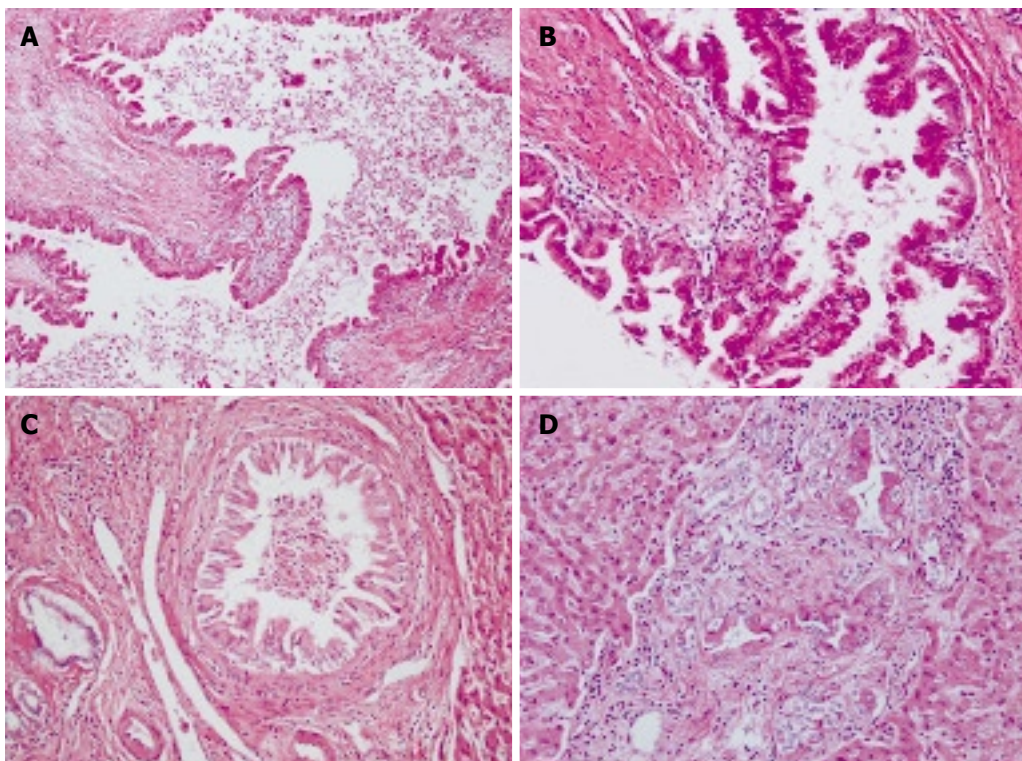


Figure 5 Histopathological findings showing. **A:** A view of the intrahepatic bile ducts dilated with peri-ductal fibrosis (x 100); **B:** Carcinoma cells continuously proliferating from the intrahepatic large bile ducts to small bile ducts (x 400); **C:** Carcinoma cells continuously proliferating from the intrahepatic large bile ducts to small bile ducts (x 200); **D:** Carcinoma cells in bile ductules around the small portal area (x 200).

extensive intraductal spreading. The histopathological stage of the tumor according to the General Rules for Surgical and Pathological Studies on Cancer of Biliary Tract was the final stage I (pT1, pN0, P0, H1, M0) and final curability was evaluated as A. No event has been observed during the medical follow-up (an 18-mo period).

DISCUSSION

IPN-B is characterized by intrabiliary papillary growth, overproduction of mucin, multifocal occurrence, jaundice and cholangitis, and also has been reported to be accompanied by hepatolithiasis^[1,2], which is recognized as being a risk factor for ICC. Recently, it has been reported that the number of cases of IPN-B without hepatolithiasis is much higher than those with it, and that it is hard to diagnose ICC with hepatolithiasis before laparotomy^[16,17].

In our case, when dilatation of the intrahepatic bile duct in the right anterior segment was detected by abdominal ultrasonography, neither a solid mass component nor hepatolithiasis was observed on imaging studies, and all laboratory data, including liver function and tumor markers (AFP, CEA, CA19-9), were within normal ranges. Therefore, we had difficulty in determining whether this case was malignant.

Several types of primary liver tumor with apparent cystic changes were considered in the differential diagnosis of our case^[18]. For instance, biliary mucinous cystadenocarcinoma, intraductal neoplasm of liver (IPNL) and cholangiocarcinoma arising in a congenital cystic liver fibrosis are known examples.

We performed ERCP to evaluate the cause of intrahepatic cholangiectasis. This showed an obstruction of the right bile duct at the root of the right hepatic duct,

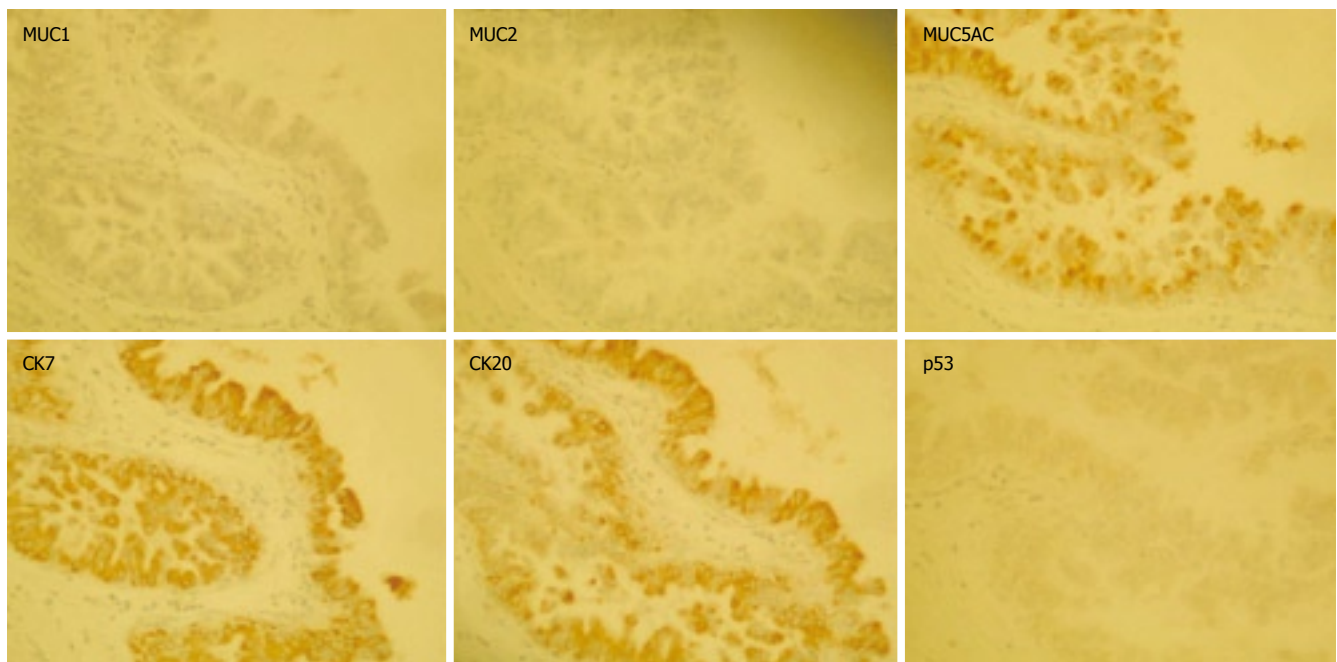


Figure 6 Immunohistological staining: MUC5AC, CK7 and CK20 are expressed in the tumor cells, whereas MUC1, MUC2 and p53 are not.

which might have been due to a mucinous plug. ERCP is recommended to determine the presence and location of suspected intraductal tumors before laparotomy^[19].

Furthermore, we believe that cholangiography is a reliable procedure for investigating the cause of bile duct dilatation and the image showed abnormalities of the bile ducts that were strikingly suspicious of the infiltration of carcinoma cells along the intrahepatic biliary epithelium. These results demonstrated that cholangiographic images are helpful in diagnosing IPN-B and determining the extent of dissection in case of surgical treatment. Generally, the prognosis of ICC (especially the non-papillary type) at an advanced stage is poor, whereas surgical excision of IPN-B has a good prognosis^[13,20]. Early detection of cancer in IPN-B improves survival, although it is hard to make an accurate assessment of the tumor invasion at an early stage, when a curative excision can be performed. As mentioned above, ERCP and cholangiography are useful for the diagnosis *in situ* carcinoma such as our case besides CT and MRI. Otherwise, bile cytology or biopsy of the bile duct as complementary diagnostic tools may be helpful to diagnose malignancy. It is necessary to cumulate the number of IPN-B cases and to observe their clinical manifestations and long term survival in advanced stage, compared to ICC.

We observed some remarkable findings in terms of histopathology. First, we needed to distinguish IPN-B from BillN. In general, the former is characterized by flat or low papillary growth of atypical biliary epithelium, whereas the latter is characterized by papillary growth of that tissue. In our case, prominent papillary growth of atypical biliary epithelium was predominant, and this was appropriate for the diagnosis of IBN-B. As mentioned above, it is said that IPN-B could be the counterpart of pancreatic IPMN, and it is divided into various clinical stages: adenoma, carcinoma *in situ* (CIS) and periductal

infiltrating type^[9,10]. There are a lot of similarities in terms of the clinicopathological findings. This case corresponds to CIS, because dysplasia of biliary epithelium was mild in the small portal vein area and severe in the large bile duct. Secondly, regarding the dilatation of the bile duct, we had to distinguish the dilatation with tumor from focal Caroli's disease or congenital hepatic fibrosis, etc. However, we considered that the dilatation was associated with a tumor because we were not able to find evidence of disease in other areas. Most IPN-Bs are characterized by mucin overproduction and dilatation of bile duct with a remarkable papillary mass, but these are not detected in some cases comparable to ours. Finally, low papillary carcinoma cells extended superficially along the intrahepatic bile ducts, which was a very interesting feature.

Zen *et al* reported various expression patterns of MUCs and cytokeratins in neoplastic biliary epithelia of BillN and IPN-B with progression to ICC in hepatolithiasis^[21]. IPN-B with hepatolithiasis was characterized predominantly by the intestinal phenotype (MUC2+/CK20+). However, in the present case, the immunophenotype was of the MUC2-/CK20+/MUC1-/MUC5AC+/CK7+ pattern, which corresponds to that of the pancreaticobiliary type^[22]. This type consists of carcinoma cells, including CIS but not adenoma, and is likely predominant in such a case without hepatolithiasis. According to recent analyses, biliary papillary tumors are characterized by the common expression of MUC2, CDX2 and CK20, and CK7 is expressed in neoplastic lesion of biliary papillary tumors^[23-25]. Identification of immunophenotypes of MUCs and CKs may aid following up patients in terms of progression.

Although there are some reports of carcinogenesis of ICC arising from IPN-B or IPNL, the carcinogenic pathways in IPN-B are still unclear. However, it has been reported recently that the state of field cancerization may

affect the carcinogenesis^[26]. It is proposed to investigate the phenotypic and genetic changes in IPN-B for improving diagnosis and therapy.

Recently, it has been reported that highly invasive IPN-B may be involved in cases of ICC at an advanced stage, although IPN-B is generally characterized by lower malignancy and well differentiated histology. Further analyses of cases are necessary to establish its clinical features, therapy and prognosis.

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CASE REPORT

Protein-losing enteropathy associated with rotavirus infection in an infant

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Abstract

Rotavirus is an acute enteric pathogen in infants and children. We reported a rare case of a 6-mo-old infant with protein-losing enteropathy (PLE) caused by rotavirus gastroenteritis, and evaluated the immunological profile in peripheral blood lymphocytes. Laboratory examinations showed lymphopenia, hypoproteinemia, hypoalbuminemia, hypogammaglobulinemia, and elevation of alpha-1-antitrypsin (α 1-AT) clearance. Lymphocytes subpopulation study revealed the reversal of CD4+/CD8+ ratio with the selective decrease of CD4-positive lymphocytes. Moreover, the excessive increase of T cells producing IFN-gamma (IFN- γ) was found, which plays an important role in the protection against viral infection. The primary or secondary activation of immune system by rotavirus may influence structural integrity and vascular permeability, which may play a triggering role in protein-losing enteropathy.

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Key words: Protein-losing enteropathy; Rotavirus; Lymphocytes producing IFN- γ ; Alpha-1-antitrypsin; Reversal of CD4+/CD8+ ratio

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INTRODUCTION

Rotavirus is the most important acute enteric pathogen in children. Rotavirus infection sometimes causes severe dehydration that is characterized by vomiting and diarrhea. Unusual complications by rotavirus infection are pneumonia, aseptic meningitis, acute myositis, encephalopathy and pancreatitis^[1-3]. There are several causes of protein-losing enteropathy (PLE) in children, involving allergic enteropathy, congenital heart disease^[4-6], and gastroenteritis, lymphangiectasia. We reported a case of an infant with PLE following rotavirus gastroenteritis and investigated the phenotypic profile in this patient.

CASE REPORT

A 6-mo-old boy presented with vomiting and diarrhea for 2 d prior to admission. Subsequently, he was hospitalized because of dehydration. The physical examinations revealed normal body weight (6755 g), pretibial pitting edema, abdominal distention and hypoactive bowel sounds. Laboratory examinations showed white blood count 9500/ μ L with 12% lymphocytes, decreased protein (27 g/L)/albumin (14 g/L) with low IgG levels (< 1.9 g/L) and hypocalcemia (0.068 g/L). C-reactive protein (CRP) was normal. A stool examination on the day of admission demonstrated positive rotavirus antigen (rapid immunochromatographic test) and no eosinophils, but rotavirus antigen was not detected on d 5 of disease. No bacteria was detected in the stool culture. Alpha-1-antitrypsin (A-1-AT) clearance was elevated to 638 mL/d (normal value < 30 mL/d). A urinalysis demonstrated neither proteinuria nor hematuria. Abdominal ultrasound and computed tomography showed bowel expansion and intestinal wall thickening with a small amount of ascites. Therefore, the diagnosis of PLE associated with rotavirus gastroenteritis was made. However, Tc99m-labeled albumin scintigraphy and intestinal biopsy could not be performed due to poor state of the patient. Although we started treatment with intravenous hyperalimentation for caloric intake and extensive replacement of albumin and gamma globulin, it was difficult to keep the normal serum level of these proteins. Although vomiting stopped, white-watery diarrhea developed more than ten times per day. Periorbital edema and abdominal distention were increased, and body weight increased to 7215 g (460 g/wk). The patient was treated with intravenous dexamethasone. After the improvement of diarrhea, we initiated the

medium-chain triglycerides (MCT) milk and the symptom did not recur. About one month later, A-1-AT clearance was decreased to 43.9 mL/d, yet abdominal CT partially showed persistent intestinal wall thickening. Lymphocytes, serum protein and albumin were normalized. In the acute phase, immunophenotyping of peripheral blood lymphocytes revealed selectively decreased percentage of CD4+ cells (15.71%) with normal percentage of CD8+ cells (38.76%) and reversal of CD4/CD8 ratio (0.41). IFN- γ producing CD4+ and CD8+ lymphocytes were markedly elevated to 14.85% and 77.58%. CD19+, CD16+ and CD56+ were within normal range. One month later, immunophenotyping still revealed selectively decreased percentage of CD4+ cells (14.55%) and reversal of CD4+/CD8+ ratio (0.42), but IFN- γ producing CD4+ and CD8+ lymphocytes were decreased to 3.46% and 0.19%. Three months later, in the convalescent phase, the proportion of CD4+ lymphocytes was normalized with CD4/CD8 ratio almost 1.0. The rotavirus antibody titer determined using complement-fixation (CF) antibody was negative (1:4) at the time of admission. Five weeks later, the antibody titer increased to 1:128. Both IgM and IgG antibodies for cytomegalovirus were negative in serological test. In clinical course, no elevation of CRP occurred.

DISCUSSION

PLE is a disease characterized by a leakage of protein loss from the gastrointestinal, which results in hypoproteinemia. PLE is associated with many disorders. Although PLE is etiologically diverse, the mechanism of protein loss was classified into two groups: (a) an abnormality of the lymph system; (b) enhancement of vascular permeability and/or an abnormality of intestinal mucosal membrane. In this patient, we believed that rotavirus gastroenteritis was involved in the pathogenesis of PLE. Because rotavirus antigen was detected on the day of admission, this is not nosocomial infection. We speculated that the mechanism of protein and lymphocyte leakage resulted from enhancement of vascular permeability, which was triggered by rotavirus infection damaging intestinal mucosa. Immunological studies showed markedly increased percentage of IFN- γ producing CD4+ and CD8+ lymphocytes. IFN- γ plays a major role in the host defense against rotavirus and the development of Th1 response. Gao *et al.*^[7] reported that serum and stool levels of IL-18 and IFN- γ were increased in children with rotavirus enteritis. These cytokines might have protective effects against acute rotavirus infection at the early stage. Xu *et al.*^[8] suggested that multiple toll-like receptors might modulate the immune response in the acute phase with rotavirus infection and play a role in the activation of IFN- γ . In other reports, determination of serum cytokines (interleukin-6, interleukin-8, tumor necrosis factor- α and CRP) might be a useful in differentiating viral from bacterial gastroenteritis^[9-11]. These cytokines in patients with bacterial gastroenteritis were significantly higher than those in patients with viral gastroenteritis, especially in combination with interleukin-6 and CRP. Although we tested only CRP, it has never been elevated in clinical course. Therefore, we diagnosed our case as

viral infection and gave no treatment of antibiotics, but more studies for other cytokines will be needed. Recently, it has been reported that a high frequency of rotavirus infections may increase the risk of celiac disease autoimmunity^[12-14]. Unfortunately, we could not exclude the possibility of this disease because of no examinations for autoantibodies, including antigliadin antibodies (AGA), antitransglutaminase antibodies (TGA) and antiendomysium antibodies (EMA). Wang *et al.*^[15] described rotavirus infection induced strong inflammation and immune responses. Although treatment with steroid in PLE patients is controversial, we treated this patient with dexamethasone to control inflammation, achieving some improvement.

Previous phenotypic studies have demonstrated that the percentages of CD4+ and CD8+ cells was reduced in acute phase of rotavirus infection with diarrhea, and subsequently this proportion of T lymphocytes recovered to almost normal levels in convalescent phase^[15]. In PLE associated with cytomegalovirus, lymphocytes subpopulation showed normal percentage of T cells, B cells and NK cells. Furthermore, the CD4/CD8 ratio was normal^[16]. It is not clear why the CD4+ lymphocytes were selectively reduced in the peripheral blood in this case. In patients with PLE, Garty *et al.* described two hypotheses for selective CD4+ cells loss after Fontan procedure^[5]. The first is that CD4+ cells may have a longer half-life, leading to faster depletion from the circulation. A slower replenishment of the cells may result in fewer availability. Another hypothesis may be the selective transport of CD4+ cells. Our immunological studies of rotavirus induced PLE revealed the selective CD4+ lymphocyte reduction and the excessive inflammation. Some immune-mediated process induced by rotavirus gastroenteritis may play a triggering role in the pathogenesis of PLE, but further investigations are needed.

In summary, we reported a rare case of severe transient rotavirus gastroenteritis accompanied with PLE. Interestingly, although the reason is not clear, the immunophenotyping of peripheral blood lymphocytes in acute phase showed a markedly decreased percentage of CD4+ cells. Rotavirus infection should be considered in patients with acute and symptomatic protein loss of gastrointestinal origin.

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Melanoma of the rectum: A rare entity

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Abstract

A 41-year-old man presented with a 6-mo history of changed defecation and rectal bleeding. A 3-cm polypoid tumor of the lower rectum was found at rectosigmoidoscopy, which proved to be a leiomyosarcoma upon biopsy. Dissemination studies did not show any metastases. He was underwent to an abdomino-perineal resection (APR). Histopathology of the specimen showed a melanoma (S-100 stain positive). Two years after the resection, metastases in the abdomen and right lung were found. He died one and half years later. Primary anorectal melanoma is a rare and very aggressive disorder. According to current data, one should always perform a S-100 stain when anorectal sarcoma is suspected. A positive S-100 stain suggests the tumour to be most likely a melanoma. Subsequently, thorough dissemination studies need to be performed. Depending on the outcome of the dissemination studies, a surgical resection has to be performed. Nowadays, a sphincter-saving local excision combined with adjuvant loco-regional radiotherapy should be preferred in case of small tumors. The same loco-regional control is achieved with less "loss of function" compared to non-sphincter saving surgery. Only in the case of large and obstructing tumors an abdomino-perineal resection is the treatment of choice.

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Key words: Melanoma; Rectum; Abdomino-perineal resection; Cancer; Surgery

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INTRODUCTION

The majority of colorectal and anal malignancies are adenocarcinomas and squamous cell cancers. Despite the predominance of these neoplasms at these locations, rare histotypes of the colon, rectum, and anus occur. These histotypes include lymphoma, melanoma, diffuse cavernous hemangioma, and sarcomas, such as leiomyosarcoma or Kaposi's sarcoma.

Primary anorectal melanoma is a rare disorder. About 1% of all anorectal carcinomas are melanomas^[1,2], typically presenting in the fifth or sixth decade of life and predominantly in woman. Patients present themselves with local symptoms like rectal bleeding and a changed defecation pattern^[1-6]. Prognosis is very poor with a median survival of 24 mo and a 5-year survival of 10%^[3]. Almost all patients die because of metastases^[4]. At this moment there is no consensus on which surgical approach is favourable. The surgical procedure of choice ranges from an abdomino-perineal resection to local excision with or without adjuvant radiotherapy.

CASE REPORT

A 41-year-old man was admitted with a 6-mo history of modified defecation and rectal bleeding. His past history was unremarkable, and his family history was negative for colon and/or rectal carcinoma. He had lost some weight, despite maintaining a good appetite. By physical examination, a 4 cm nodular mass was palpated just behind the anal verge.

Rectosigmoidoscopy demonstrated a polypoid tumor with a diameter of 3 cm, involving half the circumference. Histopathological examination delineated the tumor as a leiomyosarcoma (Figure 1). MRI showed a tumor just behind the anal verge, without evidence of invasion in the sphincter, growth outside the rectum or enlarged lymph nodes (Figure 2). Abdominal ultrasonography showed no evidence for metastases, especially not in the liver. X-ray of the thorax and CT-thorax were free from any signs of metastases.

An abdomino-perineal resection was performed. The postoperative period was uneventful. Histopathology of the resected specimen showed a melanoma (S-100 stain positive) (Figure 3).

Two years after the initial diagnosis, metastases in the abdomen were found with growth into the second lumbar vertebra and in the right lung. The patient was treated with radio-chemotherapy with good locoregional response. He underwent a metastasectomy of the right lung following

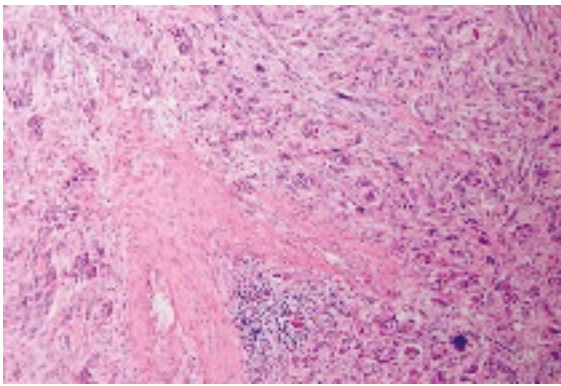


Figure 1 Histopathological examination of a biopsy taken at rectosigmoidoscopy showed the tumour to be a leiomyosarcoma (HE, $\times 100$).

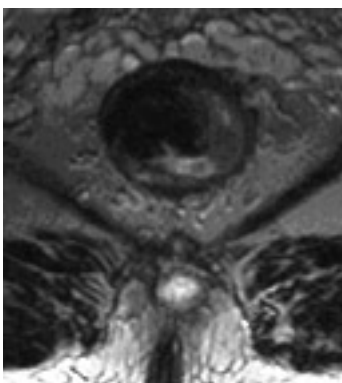


Figure 2 MRI-rectum showing a tumour just behind the anal verge.

radiochemotherapy. The postoperative period was uneventful and the disease seemed to be in remission. Three and a half year after the initial diagnosis, however, he died because of intra-abdominal metastases.

DISCUSSION

A malignant melanoma of the rectum is a rare and very aggressive rectal disorder^[1-6]. This disease presents itself usually starting from the fourth decade, predominantly in women, with an increase of incidence in the fifth or sixth decade of life^[5]. Almost 60% of patients have already metastases at initial diagnosis^[2]. Patients often present with rectal bleeding and tenesmus^[1-6].

If a biopsy shows a specimen suspicious for sarcoma (e.g. leiomyosarcoma), one should be alert. Preferably, S-100 staining should be performed in addition to routine stainings. A positive S-100 stain suggests the tumour most likely to be a melanoma. Subsequently dissemination studies, including chest X-ray and CT-scan of chest/abdomen/pelvis are performed. Curative surgical resection has to be performed in the absence of metastases proven by dissemination studies.

There is no consensus at this moment on which surgical approach is preferred.

A number of studies claim that an abdomino-perineal resection (APR) is the treatment of choice^[7,8]. This is based on the hypothesis that the disease spreads proximally via the submucosa to the mesenteric lymph nodes. Retrospec-

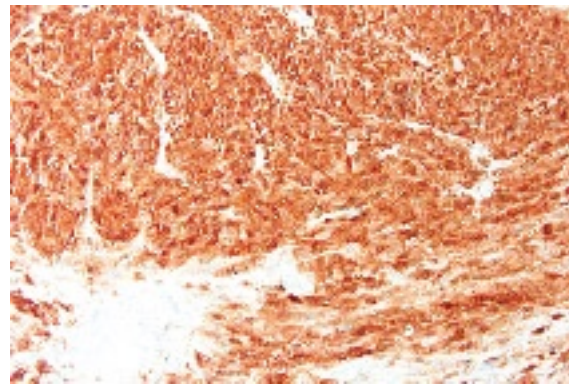


Figure 3 Histopathological examination of the abdomino-perineal resection (S-100 Positive, $\times 200$). S-100 staining performed with an automatic system (Benchmark XT, Ventana Systems). Diffuse expression of S-100 staining typical for melanoma. In leiomyosarcoma, S-100 is never expressed.

tive studies have revealed a statistically significant improvement in local-regional control when patients are managed with APR compared with local excision alone (without radiotherapy) (74% *vs* 34%)^[5].

Other studies, however, have recommended only a sphincter-saving excision for two reasons. Firstly, treatment is often palliative and wide radical surgery unnecessary mutilating. Secondly, tumor stadium and biological behaviour of the tumor determines survival instead of the choice of surgical operation^[9,10].

Recent studies show that sphincter-saving local excision combined with adjuvant loco-regional radiotherapy at the primary site of the tumor and the regional pericolic and inguinal lymphatics (5 X 6 GY) results in the same loco-regional control with less loss of function compared to APR (70% *vs* 74%)^[5].

Local excision seems to be the treatment of choice in case of small tumors, because of lower morbidity compared to APR. Only in the case of large and obstructing tumors, an APR is the surgical treatment of choice.

In conclusion, a malignant melanoma of the rectum is a rare disorder. The prognosis is poor and patients often have already metastases at the time of diagnosis. If a biopsy shows a sarcoma, the S-100 stain should be performed in addition to routine stainings. A positive staining strongly suggests a melanoma. The first choice, among surgical treatments, seems to be local excision with adjuvant radiotherapy. Only in the case of large and obstructing tumors one should perform an abdomino-perineal resection.

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www.easl.ch/hepatitis-conference

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 8th International Conference on New Trends in Immunosuppression and Immunotherapy
www.kenes.com/immuno

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

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 British Society of Gastroenterology Annual Meeting
 E-mail: BSG@mailbox.ulcc.ac.uk

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

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

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

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

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

April 23-27, Milan, Italy
 43rd Annual Meeting of the European

Association for the Study of the Liver
www.easl.ch

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

May 18-21, San Diego, California, USA
 Digestive Disease Week 2008
 June 4-7, Helsinki, Finland

The 39th Nordic Meeting of Gastroenterology
www.congex.com/ngc2008
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 Email: meetings@imedex.com

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

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

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- 5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ, Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764]

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- 6 21st century heart solution may have a sting in the tail. *BMJ*

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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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^[1]Passed away on October 20, 2007

^[2]Passed away on June 11, 2007

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Novel therapeutic approaches for hepatocellular carcinoma: Fact and fiction

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Abstract

Hepatocellular carcinoma (HCC) is the most common primary liver cancer and accounts for 80%-90% of this class of malignancy. So far, understanding of its pathogenesis and effective therapeutic methods are rather limited. In this issue, 11 invited review articles are published to address current advance of underlying molecular mechanisms for the development of HCC, and novel therapeutic approaches for HCC. This series of review articles provide an in-depth understanding of HCC that has led to or may lead to the development of novel therapies for HCC.

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Liver cancer is the fifth most common cancer worldwide and the third most common cause of cancer mortality^[1]. Hepatocellular carcinoma (HCC), which accounts for 80%-90% of primary liver, is characterized by a very poor prognosis and is associated with high mortality^[1-3]. It has been well known that chronic viral hepatitis B and C infections are the most important risk factors, responsible for 80% of HCC worldwide^[1-3]. However, current available therapeutic modalities for HCC are largely inadequate. Surgical approaches such as resection and transplantation

are the treatment of choice for HCC; however, because of underlying liver disease, only a minority of patients are suitable for resection, and access to transplantation is limited by organ availability. Local tumor ablation is effective for early HCC, and chemoembolization is of benefit in intermediate-stage disease. So far, no first-line therapy has emerged for advanced HCC. Cytotoxic chemotherapy has proven ineffective^[3]. Therefore, research efforts are focused on novel targeted therapies.

In this issue of the *World Journal of Gastroenterology*, 11 invited review articles are published to reflect the current advance in understanding the etiology of HCC and underlying molecular mechanisms for the development of HCC, and, particularly, to provide expert opinions and new insights on the potential novel therapeutic approaches for HCC.

But *et al* update the incidence and describe different patterns and risk factors for HCC, speculate on carcinogenesis in hepatitis B virus (HBV), and hepatitis C virus (HCV), point out the clinical difference between HBV- and HCV-related HCC, and describe the natural history of HCC, prediction factors for survival, and HBV vaccination^[4]. Macdonald *et al* emphasize that the incidence of HCC in patients with immunodeficiency virus (HIV) is increasing, and HCC in HIV almost invariably occurs in the context of HBV or HCV co-infection. Moreover, human HIV co-infection seems to accelerate disease progression and reduces the efficacy of anti-HBV and anti-HCV therapy^[5]. Further, Macdonald *et al* point out updated information on the screening, prevention and treatment of HCC in patients with HIV in the HAART (highly active antiretroviral therapy) era^[5]. The knowledge on HCC etiology and epidemiology is of significant value in guiding the clinical practice including diagnosis, prevention and treatment.

Various treatments options have been under investigation in order to achieve the greatest survival benefit with the least toxicity. Salem *et al* highlight the use of segmental infusion of intra-arterial radiotherapy with Yttrium-90 (Y90) or Phosphorus-32 (P-32) for the treatment of inoperable HCC and update the recent clinical and research advancements in radiotherapy^[6]. Maruyama *et al* discuss the increasingly important roles of ultrasound (US) in the diagnosis (e.g. US-guided needle puncture, color Doppler US, and real-time 3-dimensional US images), and in the treatment of HCC (e.g. high intensity focused ultrasound)^[7].

Many studies have been carried out with the aim of developing a systematic treatment and/or achieving targeted therapy at the molecular level. Silencing HCC-related cellular oncogenes or the HBV and HCV viruses has been attempted for HCC treatment. Many studies have

demonstrated promising results, and an early clinical trial assessing RNAi-based HBV therapy is currently in progress. However, there are several significant hurdles that need to be overcome before the goal of RNAi-based therapy for HCC is realized. This aspect has been well covered by Arbuthnot *et al*^[8]. Di Maio *et al* review the association between sex hormones and HCC tumorigenesis and the efficacy of anti-hormone therapy. Although epidemiological and pre-clinical studies support a strong association, several clinical trials have virtually produced negative results. Thus, there is no robust evidence that HCC is a hormone-responsive tumor, and hormonal therapy should not be a part of current management for HCC^[9]. Breuhahn and Schirmacher focus on the alterations in the insulin-like growth factor (IGF)-II signaling pathway and *in vivo* models that support the central role IGF-II signaling during HCC development and progression^[10]. This pathway has become the center of interest as a target for potential anti-cancer therapy in many types of malignancies. Therefore, inhibitors targeting IGF-IR and other RTKs or combinations of different specific substances targeting distinct pathways might be attractive therapeutic approaches for HCC in the future. The contribution by Deli *et al* reviews the alterations in components of the activin signaling pathway that have been observed in HCC and discuss their potential significance for liver tumorigenesis^[11]. Activin A, and possibly activin E, may have a similar tumor suppressive function in the liver as TGF- β although whether activins may also shift to a pro-tumorigenic function during tumor progression is little explored. Activin antagonists may serve to block the growth inhibitory and pro-apoptotic activity of activin A on hepatocytes. Therefore, a targeted inhibition of activin antagonists might restore sensitivity to activin-induced growth inhibition and apoptosis, and may thus represent a feasible strategy to inhibit tumor growth. Future studies will clarify whether such approaches may offer new therapeutic opportunities for combating liver cancer^[11].

The review of Martin and Dufour address the various tumor suppressors which have been implicated in HCC^[12]. Specifically, they discuss the function of these tumor suppressors and the related signaling pathways such as p53, Wnt, Ras/Jak/Stat and other pathways and the involvement of inactivation of these tumor suppressors in the initiation or progression of HCC as well as the underlying mechanisms of their inactivation^[12]. Matsuda provides evidence that p16 and p27, two potent tumor suppressors that inhibit cyclin-dependent kinase, are functionally related, i.e. loss of p16 expression is associated with over-expression but functional inactivation of p27 in HCC. In addition, loss of p16 expression is an independent prognostic factor for a poor outcome in HCC cases expressing high levels of p27. Thus, p16 and p27 may become more accurate biomarkers predicting the prognosis of HCC^[13]. Moreover, comprehensive understanding of the functions of and interactions among these tumor suppressors and the underlying mechanisms of their inactivation is a prerequisite to design innovative treatments of HCC.

It has been well known that development of HCC

is a multistep process with accumulation of genetic and epigenetic alterations in regulatory genes, leading to activation of oncogenes and inactivation or loss of tumor suppressor genes (ref. above). Tischoff *et al* further discuss the epigenetic alterations in HCC focusing on DNA methylation^[14]. In HCC, aberrant methylation of promoter sequences occurs not only in advanced HCC, but also in premalignant conditions such as chronic viral hepatitis B or C and hepatic cirrhosis. Therefore, epigenetic changes in preneoplastic or early neoplastic stages may serve as an indicator or "biomarker" for screening of patients with an increased risk for HCC. Moreover, it has been demonstrated that reexpression of TSGs that are epigenetically silenced is possible by using demethylating and histone modifying agents, indicating a potential therapeutic approach by specifically modulating DNA hypermethylation^[14].

It is our hope that this series of review articles will give our readers an in-depth understanding of all aspects of HCC that have led to or may lead to the development of novel therapeutic approaches for HCC.

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Complications of collagenous colitis

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Abstract

Microscopic forms of colitis have been described, including collagenous colitis. This disorder generally has an apparently benign clinical course. However, a number of gastric and intestinal complications, possibly coincidental, may develop with collagenous colitis. Distinctive inflammatory disorders of the gastric mucosa have been described, including lymphocytic gastritis and collagenous gastritis. Celiac disease and collagenous sprue (or collagenous enteritis) may occur. Colonic ulceration has been associated with use of nonsteroidal anti-inflammatory drugs, while other forms of inflammatory bowel disease, including ulcerative colitis and Crohn's disease, may evolve from collagenous colitis. Submucosal "dissection", colonic fractures or mucosal tears and perforation from air insufflation during colonoscopy may occur and has been hypothesized to be due to compromise of the colonic wall from submucosal collagen deposition. Similar changes may result from increased intraluminal pressure during barium enema contrast studies. Finally, malignant disorders have also been reported, including carcinoma and lymphoproliferative disease.

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Key words: Colonic fractures; Colonic perforation; Celiac disease; Collagenous gastritis; Collagenous sprue; Cat scratch colon; Colon cancer; Microscopic colitis

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COLLAGENOUS COLITIS

Collagenous colitis was first described just over 3 decades ago and usually presents as a watery diarrhea syndrome^[1]. Distinct histopathologic changes consisting of a colonic mucosal inflammatory process with a characteristic subepithelial hyaline deposit in the lamina propria region (Figures 1 and 2) have been described^[1]. These pathological features are analogous to those reported earlier in collagenous sprue^[2]. Ultrastructural studies have confirmed that the deposits consist of collagen fibers^[3].

Most often, middle-aged to elderly females are affected, although even children have been reported with collagenous colitis^[1,4]. The cause has not been defined, but the disorder is very heterogeneous and may have multiple causes. It has frequently been associated with celiac disease^[5] and use of a broad range of medications, including nonsteroidal anti-inflammatory drugs and proton pump inhibitors, i.e., lansoprazole^[1]. Moreover, there are familial cases suggesting that genetic or heritable factors play a critical role^[6]. There are also reports suggesting that collagenous colitis may be precipitated by enteric infections, such as *Yersinia* species or, possibly, bacterial toxins^[7-9]. In addition, collagenous colitis has appeared as a reversible paraneoplastic phenomenon^[10]. Finally, collagenous colitis has also been recorded in non-human species, such as the baboon^[11]. Collagenous colitis may also be readily distinguished from other forms of inflammatory bowel disease (Table 1).

Spontaneous resolution may occur so that evaluation of the response to different therapies may be made more difficult. Even histological endpoints are difficult to evaluate because the sub-epithelial collagen deposits tend to be patchy, rather than diffuse and continuous in mucosal distribution^[1]. Treatment has most often focused on symptom resolution using added dietary fiber, non-specific anti-diarrhea agents and anti-inflammatory medications. Steroids, specifically budesonide, were shown to be useful in clinical trials^[12]. In some, other immunosuppressants have been used and even surgical treatment has been described^[13]. Of note, ileostomy and sigmoidostomy were reported to lead to both clinical and histologic remission. Later ostomy closures, however, led to recurrent symptoms and re-development of collagen deposits. Possibly, a diverted noxious luminal factor was important^[14]. Finally, collagenous pouchitis has also been described after restorative proctocolectomy^[15].

Long-term studies of collagenous colitis have suggested that it generally runs a very benign clinical course, at least

Table 1 Comparative features of collagenous colitis (CC) with ulcerative colitis (UC) and Crohn's disease (CD)

	CC	UC	CD
Usual age at diagnosis	Mid to older age, most over 40	Youth to middle age	As for UC, most under 40
Sex predominance	Usually female	Similar	Similar
Diarrhea	Watery	Usually bloody	Watery, sometimes with blood
Distribution in colon	Patchy, rare rectal sparing	Continuous with rectal involvement	Patchy or focal; some with rectal sparing
Mucosal involvement	Yes, with	Yes	Yes, with granulomas
Small bowel disease	Occurs; also celiac disease	"So-called 'reflux' ileitis with severe pancolitis"	Common, especially ileum
Complicating cancer	To date, no increased frequency	"Increased rates with extensive disease"	"Increased rates with extensive disease; small bowel cancer"
Autoimmune disorders	Yes	Yes	Yes

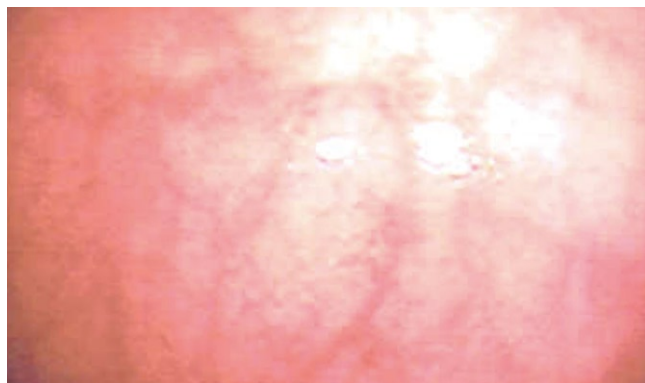


Figure 1 Gross macroscopic appearance of colonic mucosa in collagenous colitis. During colonoscopy, the mucosa may appear virtually normal with minimal alteration in the clarity of the vascular pattern. Diagnosis depends on microscopic evaluation of a colonic biopsy, not the macroscopic appearance of the colonic mucosa.

during evaluation over a period of about 10 years^[16]. In most, symptoms spontaneously resolved or remission occurred in association with anti-inflammatory drug therapy alone. Some experienced persistent diarrhea or intermittent periods of recurrent diarrhea that required on-going chronically-administered medication. Occasionally, however, collagenous colitis may be complicated by other disorders of the stomach, small or large intestine and these may dominate its clinical course.

COMPLICATIONS

In a prospective evaluation of established collagenous colitis, celiac disease was subsequently detected in over 20%^[5]. This could have implications for patients with celiac disease not appearing to respond to a gluten-free diet; in these, the colonic disease, rather than celiac disease, might be causing symptoms. Collagenous involvement of the gastric and/or small intestinal mucosa also has been detected with collagenous colitis suggesting that, in some, histological changes may not be simply localized in the colon, but may be a far more extensive process^[10,17,18]. Even with extensive collagenous involvement of the intestinal tract, however, complete histological remission is possible^[10,18].

While most patients with collagenous colitis have a benign or sometimes relapsing and remitting course, recent reports have increased appreciation for some of its possibly more unusual clinical features involving the colon. A severe and protracted course with a fatal outcome

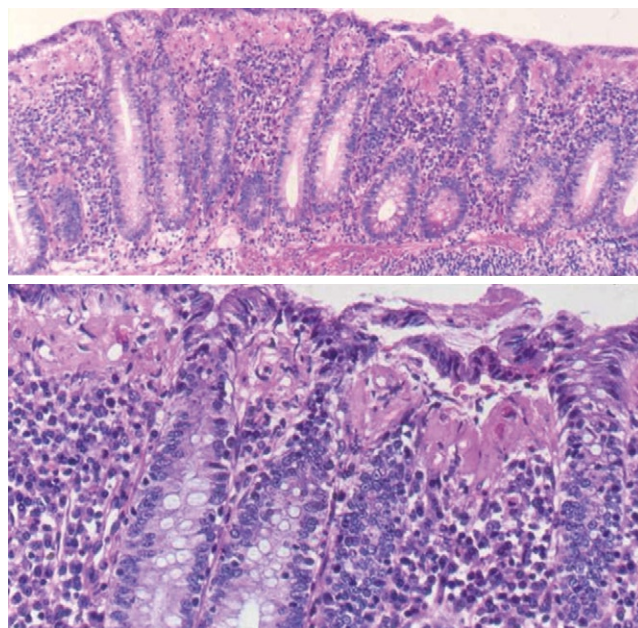


Figure 2 Lower and higher power photomicrographs of the colonic mucosa in collagenous colitis. A mucosal inflammatory process is present with well preserved crypt architecture. Note the subepithelial mucosal band of collagen material.

attributed to the colitis has been recorded^[19]. Surface epithelial cell sloughing may be appreciated in colonic biopsies leaving a naked subepithelial deposit; in many of these patients, mucosal permeability may be altered and protein-losing enteropathy has been noted in the absence of small intestinal disease^[20]. Colonic ulceration may occur, possibly related to concomitant use of medications, such as nonsteroidal anti-inflammatory drugs^[21]. Occasionally, evolution of collagenous colitis into severe ulcerative colitis or Crohn's disease has been recorded^[22-24]. With the onset of ulcerative colitis, complete disappearance of the collagen deposition occurred^[22]. Rarely, submucosal "dissection" may occur^[25]. Colonic fracturing after endoscopic instrumentation, possibly related to air insufflation and barotrauma, or insertion of barium contrast agents, has been recorded^[26]. Recently, a report entitled "cat scratch colon" emphasized the macroscopic changes involving the proximal colon that are most often observed during colonoscopy in collagenous colitis^[27]. Spontaneous peritonitis with colonic perforation has also been recorded^[28]. In all of these, it has been hypothesized that the integrity of the colonic wall may be compromised owing to submucosal collagen deposition.

A particularly striking finding, to date, is the rarity of reported malignant disease complicating the clinical course of collagenous colitis. Colorectal cancer has been noted^[10,29], including cecal cancer^[30], but these may have been only coincidental. In an extensive survey study, the overall risk of colorectal cancer was similar to the general control population; only 2 patients with collagenous colitis were seen with colorectal cancer, but these occurred before development of colitis^[31]. Interestingly, collagenous involvement of the small and large intestine resolved completely following resection of a colon cancer suggesting an unusual paraneoplastic phenomenon^[10]. Other neoplasms that have been rarely recorded include lymphoproliferative disorders^[32,33] and carcinoids^[34]. Because this is a relatively "new" disease, sufficient time may not yet have passed to observe sufficient superimposed disease complications. Alternatively, factors linked to pathogenesis, such as the use of nonsteroidal anti-inflammatory drugs, may induce a chemopreventive effect on development of colonic neoplasia.

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REVIEW

Gastric cancer: Animal studies on the risk of hypoacidity and hypergastrinemia

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Abstract

Gastric hypoacidity and hypergastrinaemia are seen in several conditions associated with an increased risk of gastric malignancy. Hypoacidity and hypergastrinaemia are closely related and their long-term effects are difficult to study separately in patients. Studies using animal models can provide valuable information about risk factors and mechanisms in gastric cancer development as the models allow a high degree of intervention when introducing or eliminating factors possibly affecting carcinogenesis. In this report, we briefly review findings from relevant animal studies on this topic. Animal models of gastric hypoacidity and hypergastrinaemia provide evidence hypergastrinaemia is a common causative factor in many otherwise diverse settings. In all species where sufficient hypoacidity and hypergastrinaemia have been induced, a proportion of the animals develop malignant lesions in the gastric oxyntic mucosa.

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Key words: Gastrin; Gastric cancer; Proton pump inhibitors; Acid secretion; Animal model

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INTRODUCTION

Gastric hypoacidity and subsequent hypergastrinaemia

occur in patients with parietal cell loss due to atrophic gastritis as well as in patients using drugs inhibiting gastric acid secretion. The increased risk of developing gastric carcinoids as well as adenocarcinomas is well documented in patients with pernicious anemia^[1-4], whereas there is no direct evidence that hypoacidity and hypergastrinaemia caused by proton pump inhibitors promote gastric carcinogenesis in humans. Proton pump inhibition for up to five years has led to enterochromaffin-like (ECL) hyperplasia related to hypergastrinaemia^[5]. Patients with gastrinomas often develop ECL cell carcinoids secondary to hypergastrinaemia, as well as signet ring cell carcinoma^[6]. ECL cell carcinoids develop more often in patients with multiple endocrine neoplasia type 1 (MEN1)^[7], but are also found in patients without MEN1^[8,9]. The common factor in the above mentioned conditions is prolonged hypergastrinaemia, and it has been presumed that hypergastrinemia in itself increases the risk of gastric neoplasia in humans^[10,11].

However, gastric carcinogenesis in humans is a process that progresses over years to decades and may be influenced by numerous factors. Dissecting the mechanisms of gastric carcinogenesis in humans is therefore difficult and several animal models have been used to reduce the number of variables affecting this process. Various animal models have demonstrated that regulation of acid secretion and oxyntic mucosal growth are interwoven and animal models have provided detailed knowledge regarding the role of hypoacidity as well as hormones and signal substances in gastric carcinogenesis. In this report, we briefly review findings from animal studies on the role of gastric hypoacidity and hypergastrinaemia in gastric carcinogenesis.

GASTRIN AND THE GASTRIN RECEPTOR

Gastrin, which stimulates gastric acid secretion, was the second hormone to be postulated to exist^[12]. The release of gastrin from G cells is inhibited by hydrochloric acid. Gastrin exerts its effect on the CCK-B receptor, which was first localised in the brain^[13], but later found to be identical to the gastric gastrin receptor^[14]. Gastrin regulates gastric acid secretion through a cellular sequence in which gastrin stimulates ECL cells to secrete histamine, which in turn stimulates secretion of hydrochloric acid from parietal cells^[15,16]. However, parietal cells have also been reported to express gastrin receptors^[17,18] and whether or not these are functional has been debated. Very high concentrations of gastrin potentiates histamine-stimulated acid secretion from isolated parietal cells^[19], suggesting the existence of a functional gastrin receptor on parietal cells. However,

fluorescein-labeled CCK-8 binds to rat ECL cells, but not to parietal cells^[20], and gastrin does not stimulate acid secretion in either histidine decarboxylase (HDC)-deficient^[21] or H2 receptor-deficient^[22] mice. Altogether, this implies the physiological effect of gastrin is fully mediated by histamine secreted by the ECL cell and that only the gastrin receptors on ECL cells are of physiological importance. These observations are also relevant to understanding the trophic and carcinogenic effects of gastrin on the oxyntic mucosa.

GASTRIC HYPOACIDITY, HYPERGASTRINEMIA AND GASTRIC NEOPLASIA

Gastrin was shown early to have a general trophic effect on the oxyntic mucosa^[23]. In 1985 it was first published that rats develop ECL cell carcinoids after lifelong inhibition of gastric acid secretion by administration of the long-acting insurmountable histamine 2 receptor antagonist loxidine^[24]. At the time, it was speculated whether this effect was specific for one drug, but the following year it was found that life-long administration of the proton pump inhibitor omeprazole also caused hyperplasia of the oxyntic mucosa and carcinoids in rats^[25]. Even the short-acting histamine 2 receptor antagonist ranitidine was later shown to cause ECL cell carcinoids in 19 of 100 rats after 2-year of administration, and these animals showed a 3-fold increase in plasma gastrin^[26].

Short-term administration of omeprazole (400 $\mu\text{mol/kg}$) to rats caused a 15-fold increase in plasma gastrin as well as hyperplasia of the oxyntic mucosa^[27-29]. The ECL cell density was tripled after 10 wk, whereas the total oxyntic mucosal thickness increased 20%^[28].

Hypoacidity and hypergastrinaemia are closely related in the normal state, but the effects of each factor can be studied separately in several animal models. Infusion of gastrin has been mentioned and is feasible for short-term proliferation studies in which the specific proliferative effect on ECL cells has been documented^[30-32].

Low pH in the antrum inhibits gastrin release, and, by removal of a large proportion of the oxyntic mucosa by partial corpectomy, the antral pH is raised and gastrin release is stimulated. Rats develop carcinoids in the remaining 25% of the oxyntic mucosa^[33] demonstrating that acid-inhibiting drugs per se do not cause neoplasia, but antral hypoacidity and subsequent hypergastrinaemia. Furthermore, oral administration of ciprofibrate, which is a peroxisome proliferator and a hypolipidemic compound, induces hypergastrinemia and carcinoid formation after 2 years in rats^[34]. Ciprofibrate does not cause gastric hypoacidity^[35], but induces hypergastrinemia through a direct effect on the antral G cell^[36]. Altogether, there is evidence that hypergastrinaemia induced by either method, whether accompanied by gastric hypoacidity or not, causes ECL cell carcinoids in rats.

After carcinoid development due to hypergastrinemia was demonstrated in the rat, similar experiments were performed in mice to examine possible species differences. Administration of loxidine to mice at various doses for

2 year induced carcinoid tumors of the gastric corpus^[37], whereas long-term studies with proton pump inhibitors in mice have been inconclusive, as mice require much higher doses of proton pump inhibitors than rats to maintain a high gastric pH^[38].

Transgenic (INS-GAS) mice over-express gastrin and have a 4-fold increase in plasma gastrin at age 6 mo, which leads to an increased gastric acid secretion^[39]. In this animal model it is possible to study the effects of hypergastrinemia without gastric hypoacidity, mimicking human gastrinomas. Young INS-GAS mice have an increased ECL cell number and a proportion of these mice develop adenocarcinomas in the gastric corpus at the end of their lifespan^[39]. Inoculation with *Helicobacter felis* increases gastrin levels 7-fold and accelerates carcinogenesis considerably^[39], but mice without *Helicobacter* infections also develop carcinomas, demonstrating that carcinogenesis does not depend on infection and inflammation. The reason why INS-GAS mice develop malignancy in the oxyntic mucosa with an adenocarcinoma phenotype, whereas mice and rats develop ECL cell carcinoids after long-term acid inhibition or ciprofibrate administration, is not known. A synergistic inhibitory effect of gastrin and histamine receptor antagonists on hypergastrinemia-driven carcinogenesis has been found in INS-GAS mice^[40], suggesting a role for both gastrin and histamine, but leaves questions regarding the cellular location of the histamine 2 receptor, and thus, the cellular origin of the adenocarcinomas. It has been found *H. pylori* lipopolysaccharides stimulate ECL cell proliferation and secretion in the rat^[41], supporting a concept of synergism of gastrin and *H. pylori* infection on ECL cell growth.

INS-GAS mice have been inoculated with a *H. pylori* strain and only inoculated males developed gastric cancer, whereas serum gastrin concentrations did not differ between the sexes^[42].

HDC-deficient mice show gastric hypoacidity and a threefold increase in plasma gastrin levels^[21]. This model has been mainly used for studying acid secretion, and long-term studies examining the possible carcinogenetic effects of hypergastrinemia in the absence of histamine have not been published to date. However, in animals aged 8 to 12 wk there was no difference in mucosal thickness^[21].

Another genetically modified mouse model is $\text{H}^+\text{K}^+\text{ATPase}$ beta subunit-deficient mice. These mice are anacidic, show a 7-fold increase in serum gastrin levels and hyperplasia in the oxyntic mucosa^[43,44]. $\text{H}^+\text{K}^+\text{ATPase}$ -deficient mice have been followed for up to 14 wk only, and changes in the gastric mucosa in old mice have not been published. Gastrin and $\text{H}^+\text{K}^+\text{ATPase}$ double knock-out mice are anacidic without gastrin and do not develop hypertrophic changes in the oxyntic mucosa, demonstrating that gastrin is responsible for these changes^[43].

Gastrin-deficient mice show no basal acid secretion^[45] and provide a model for studying the effect of gastric hypoacidity without hypergastrinemia. Gastrin-deficient mice develop antral adenocarcinomas^[46] through a mechanism that must be different from carcinogenesis in the oxyntic mucosa in hypoacidic and hypergastrinemic mice. The development of carcinomas in the absence of gastrin is attributed to bacterial overgrowth and subsequent formation

of carcinogenic substances^[47,48]. However, gastric hypoacidity is also found in H⁺K⁺ATPase-deficient mice which do not develop antral carcinomas (Fossmark R *et al* unpublished observations) and it is possible the lack of gastrin itself induces the carcinomas.

GROWTH PROMOTION MEDIATED BY ECL CELLS

In the oxyntic mucosa, only ECL cells have gastrin receptors shown to have a functional role in mucosal growth regulation. Several studies mentioned have described the trophic effects of gastrin on the oxyntic mucosa, and the trophic effect on the ECL cell in particular, but less is known about the further mediation of the gastrin effect. It has been shown administration of gastrin has a proliferative effect in the neck region of the oxyntic glands^[49], where stem cells are located. However, it has not been possible to settle whether this proliferative effect is a direct effect or mediated by growth factors released by ECL cells^[50]. Several ECL cell products have been suggested to stimulate growth of the oxyntic mucosa, either on parietal cells, stem cells or ECL cells themselves.

Histamine was the first ECL cell product to be identified and has been suggested to have a trophic effect^[50], but the effects of histamine are not fully understood. Histamine has been shown to stimulate proliferation of gastric cancer cell lines^[51], but the histamine 1 receptor antagonist astemizole has an additional trophic effect when administered to rats with omeprazole-induced hypergastrinemia^[52]. Histamine 2 receptor-deficient mice are hypergastrinemic and develop hypertrophy of the oxyntic mucosa^[22], supporting previous studies suggesting the mediation of histamine's trophic effect does not involve the H2 receptor^[53]. However, in histamine decarboxylase-deficient mice there was no difference in parietal and ECL cell numbers compared with controls, in animals aged 8 to 12 wk, and no increase in oxyntic mucosal thickness^[21], indicating an important role for histamine as a mediator of hypergastrinaemia-driven mucosal growth.

The regenerating gene (Reg) cDNA was first isolated from regenerating pancreatic islets in rats and its human homologue was named RegI alpha^[54], but Reg protein is also expressed in ECL cells^[55]. Gastrin stimulates Reg protein expression in ECL cells, and Reg protein is mitogenic to gastric mucosal cells^[56], suggesting Reg protein is involved in gastrin-induced gastric mucosal cell growth. Reg expression is also increased in healing gastric mucosa^[57]. Reg protein receptors are found on parietal cells and chief cells in the lower part of the corpus glands^[58]. A recent study using INS-GAS mice has found expression of Reg1 is controlled through separate promoter elements by gastrin and *Helicobacter*^[59], implying that these factors affect carcinogenesis through Reg protein. RegI alpha protein is also found in 35% of human gastric adenocarcinomas, particularly in those that are less well differentiated^[60], and Reg protein expression is associated with higher proliferation rates in early gastric cancers^[61]. Altogether, this makes Reg protein a strong candidate for mediating the general trophic effects of gastrin on the oxyntic mucosa.

OTHER ANIMAL MODELS OF HYPOACIDITY AND HYPERGASTRINEMIA IN GASTRIC CARCINOGENESIS

A strain of Japanese cotton rats develops spontaneous carcinomas in the oxyntic mucosa with a marked female predominance^[62]. Animals developing carcinomas have gastric hypoacidity of an unknown cause and secondary hypergastrinemia^[63]. The tumors develop from an oxyntic mucosa with marked hyperplasia of chromogranin A, synaptophysin and HDC-immunoreactive cells, and a proportion of the tumor cells are chromogranin A-, pancreastatin-, HDC- and Sevier-Munger-positive^[63-66]. Carcinomas develop after 4 mo of hypergastrinemia, but are prevented by the gastrin receptor antagonist YF486^[64], demonstrating gastrin is essential in carcinoma development in cotton rats. Carcinomas can also be induced in male cotton rats by administration of the histamine 2 receptor antagonist loxidine^[67], as well as by partial corpectomy^[68], two different methods of inducing pronounced hypergastrinemia. It has also been found ECL cells in hypergastrinemic animals gradually lose ultrastructural characteristics as well as chromogranin A and pancreastatin immunoreactivity^[66] suggesting ECL cells dedifferentiate during long-term stimulation by gastrin. The cotton rat model is important as it demonstrates tumors with an adenocarcinoma phenotype, but with neuroendocrine differentiation, are induced by gastric hypoacidity and hypergastrinemia and develop through dedifferentiation of ECL cells.

In the African rodent *Mastomys*, multicentric gastric carcinoids frequently develop in the oxyntic mucosa of a proportion of aging animals. Serum gastrin levels in *Mastomys* developing tumors is normal^[69] and the development of spontaneous ECL cell tumors is most likely related to a gastrin receptor mutant which shows ligand-independent activity; that is, the receptor is constitutively activated^[70]. However, endogenous gastrin is involved in the growth of ECL cell carcinoids in *Mastomys* as the development of these tumors is significantly enhanced by loxidine-induced hypoacidity and hypergastrinemia^[71,72] and carcinoid development is inhibited by a gastrin receptor antagonist^[73]. There is no sex-difference in the occurrence of such tumors. The development of carcinoids in *Mastomys* is prevented by a somatostatin analogue^[74].

Mongolian gerbils inoculated with *H. pylori* are used extensively in research on *H. pylori*-related gastric carcinogenesis. In the context of this paper, Mongolian gerbils are interesting as they become hypergastrinemic in response to *H. pylori* infection and develop mainly ECL cell carcinoids, but also gastric adenocarcinomas^[75,76]. Inoculated animals show a five to ten-fold rise in serum gastrin, increasing with time after inoculation^[77]. An increasing proportion of the gerbils develop ECL cell hyperplasia and carcinoids from 12 to 24 mo after inoculation^[76]. Atrophic gastritis and focal intestinal metaplasia and dysplasia also appear 6 mo after inoculation, and premalignant changes can be reversed after *H. pylori* eradication^[78]. Hypergastrinemia in *H. pylori*-infected animals is associated with increased Reg gene expression, and both plasma gastrin and Reg mRNA levels are normalized after *H. pylori* eradication^[79].

Finally, the possibility of species differences in relation to carcinoid development after long-term administration of acid inhibitors has also been studied in dogs. Beagle dogs were given omeprazole daily for 7 years, but there were no changes in the gastric mucosa at termination and there were specifically no increases in ECL cell numbers^[80]. However, dogs receiving omeprazole had fasting and meal-stimulated plasma gastrin levels at the same level as controls, and changes in the oxyntic mucosa should therefore not be expected.

CONCLUSION

Animal models can provide valuable information about risk factors for gastric cancer as the models allow a high degree of intervention when introducing or eliminating factors possibly affecting carcinogenesis. The various animal models of gastric hypoacidity and hypergastrinemia provide evidence hypergastrinemia is a common causative factor in many otherwise diverse settings. In all species where sufficient hypoacidity and hypergastrinemia has been induced, a proportion of the animals develop neoplastic lesions. Bearing in mind that gastrin acts on gastrin receptors located on ECL cells, which are stimulated to secretion and proliferation, we find it obvious ECL cells have a pivotal role in the gastric carcinogenesis associated with hypergastrinemia. Findings in Japanese cotton rats and Mongolian gerbils in particular suggest carcinoids and adenocarcinomas develop through a similar mechanism, and derive from ECL cells. Hypergastrinemia induces gastric neoplasia whether accompanied by gastric hypoacidity or not, and experiments using the above-mentioned models could explain why carcinoids develop in some situations, whereas tumors with an adenocarcinoma phenotype develop in other models.

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TOPIC HIGHLIGHT

Harry HX Xia, PhD, MD, Series Editor

Natural history of hepatitis-related hepatocellular carcinoma

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Abstract

Hepatocellular carcinoma (HCC) is an important cause of cancer death in the world. It has great regional differences in the pathology and epidemiology. The variation is greatly influenced by the aetiologies of the disease. Hepatitis B and C infection are the most important risk factors. HCC incidence rates are higher but in decreasing trend in developing countries. However, the figures in the developed countries are contrary. Successful hepatitis B virus (HBV) vaccination programs, better food hygiene, increased global hepatitis C virus (HCV) prevalence and population migration are the possible explanations. A number of clinical and pathogenic differences exist between HBV- and HCV-related HCC. HBV infection leads to the development of HCC through direct and indirect pathways as it has the ability to integrate into the host genome affecting cellular signaling and growth control. HCV causes HCC mainly through indirect pathways: chronic inflammation, cell deaths and proliferation. As a result, HCC is almost exclusively found in cirrhotic HCV patients while HCC is sometimes found in HBV patients without significant liver cirrhosis. Due to the different severities of liver cirrhosis and HCC extent, therapeutic strategies from resection, liver transplantation to symptoms palliation are available. Poorly differentiated histology, lack of fibrous capsule, large tumour size, early vascular invasion and elevated serum levels of alpha fetoprotein (AFP) are the features for more aggressive disease. Combined with markers of liver reserve and performance status, accurate scoring systems and models have been developed to predict patients' survival and match best treatment option.

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Key words: Natural history; Hepatitis; Hepatocellular carcinoma

INTRODUCTION

Hepatocellular carcinoma (HCC) is the third most common cause of cancer death in the world. It results in 598 000 deaths per year worldwide. Because of its poor prognosis, this number of deaths is almost the same as the number of cases being diagnosed each year (626 000)^[1]. From a global perspective, the two most important risk factors for HCC are chronic hepatitis B and C infection. The geographic distribution patterns of HCC and hepatitis B virus (HBV) almost coincide with each other. The characteristics of patients with HCC are influenced by the etiology and the status of the underlying liver disease. The understanding of its natural history may influence the prognosis and choice of treatment.

HISTORY OF HCC

HCC was believed to be a rare disease in the early 1900s. In Germany, Eggel found only 163 cases of HCC autopsies when he surveyed the world literature for HCC. He proposed an anatomical classification that HCC was classified as nodular, massive and diffuse^[2]. However, it was found that the disease was more prevalent outside Europe. In 1911, Yamagiwa suggested a classification based on the origin of the cancer cell, hepatocellular and cholangiocellular, with the terminology of "hepatoma" and "cholangioma" respectively. After Berman's report of the extremely high HCC incidence among young Mozambican males in 1951, the interest in the studies of HCC increased. It has been shown that there is great regional difference in the pathology and epidemiology of the condition.

DIFFERENT HCC PATTERNS

HCCs in South Africa are mostly large sized and poorly

differentiated with relatively healthy surrounding liver tissues. On the contrary, HCCs in non-African countries are relatively smaller in size and more differentiated with a background of cirrhotic liver. This can be the result of the difference in aetiologies. Aflatoxin B1, a mycotoxin produced by *Aspergillus* species, is believed to be a major carcinogenic factor in the South African population. Chronic hepatitis and the resultant cirrhosis are believed to be important risk factors in non-Africans. The distinct fibrolamellar type of HCC almost exclusively found in young Caucasian patients may be due to ethnic factor.

HCC INCIDENCE

Liver cancer incidence rate varies widely from 52.1 per 100 000 in China to 5.1 per 100 000 in Northern Europe. Developing countries contribute more than 80% of cases with China alone accounting for 55%^[1]. Other areas with high incidences are sub-Saharan Africa, eastern and southeastern Asia, and Melanesia. In the past decades, liver cancer incidence has decreased in some areas with high incidence in the past such as Shanghai, Singapore and Hong Kong. However, the opposite was reported in some of the countries in Europe, North America and Oceania. It is believed that effective HBV vaccination programmes and the better control of aflatoxin exposure in the high HCC incidence areas are contributing to the decreased HCC incidence while the increasing HCC incidence in some of the western countries is attributed to the increasing prevalence of hepatitis C virus (HCV) infection and immigration of people from countries with high endemicity for HBV infection.

RISK FACTORS FOR HCC

The main established risk factors for HCC development are chronic viral hepatitis B and C infection and aflatoxin B1. Liver cirrhosis *per se* may also lead to HCC development. Hepatitis C infection probably causes HCC through the pathway of cirrhosis. Alcoholic liver disease, autoimmune liver diseases, primary hemochromatosis and Wilson's disease are also associated with the development of HCC. Overall, hepatitis B and C infections are causally associated with over 80% of HCC in the world^[3].

Worldwide, there are 400 million people infected with chronic hepatitis B, seventy five percent of whom are Asians. The natural history of chronic hepatitis B infection in Asian and African countries is different from that in the western world. In Asia and Africa, the majority of the people acquire the infection during the perinatal period or during very early childhood. There is a characteristically prolonged immune tolerance phase in the first few decades of life. The HBV viral loads are high and the liver transaminases are nearly normal. The lifetime risk of HCC in infected men is estimated to be 10% to 25% while the risk in women is somewhat lower^[3]. Case-control studies have shown that chronic HBV carriers have more than 100-fold increased risk of HCC compared with non-infected individuals. Furthermore, the risk of HCC development is related to

the disease status of chronic hepatitis B infection. For example, the estimated annual risk of HCC in chronic carriers is 0.26% to 0.6%. The risk increases to 1% in patients with active hepatitis^[4]. It further increases to 2% to 3% in cirrhotic patients. Certain viral status also poses a higher risk of HCC. Studies in Taiwan showed that genotype C chronic hepatitis B infection has a more aggressive progression than genotype B in HBeAg-positive patients^[5]. Core promoter mutations (T1762/A1764) are also found to be related to a more progressive disease^[6]. In the majority of HCC cases (70% to 90%) there is underlying liver cirrhosis. However, HBV, being an oncogenic virus, can cause HCC in the absence of cirrhosis through the pathway of integration into the human genome.

HBV CARCINOGENESIS

As with many malignancies, the carcinogenesis of HCC is a multi-step process involving a number of different genetic alterations that ultimately lead to malignant transformation of the hepatocyte. It is postulated that HBV infection causes HCC *via* direct and indirect pathways. Continuous hepatocyte injury and regeneration in cirrhosis of the liver leads to increased liver cell turnover and hence accumulation of critical mutations in the host genome. This may result in genetic alterations, such as chromosomal rearrangements as well as activation of cellular oncogenes or inactivation of tumour suppressor genes. However a higher rate of chromosomal abnormalities is found in HBV-related HCC than those linked to other risk factors^[7,8]. Alternative mechanisms in HBV carcinogenesis must also be involved. HBV belongs to the group of oncogenic viruses known as hepadna virus. It is able to integrate its DNA into the genome of the infected cell. Integrated HBV sequences have been observed in established hepatoma cell lines and in about 80% of human HBV related HCCs^[9]. It is postulated that the HBV DNA integration may confer a selective growth advantage on target cells and leads to the onset of tumor progression. The integration sites are frequently detected in cellular genes involving cell signaling or growth control. Host chromosomal instability is also enhanced by HBV DNA integration. Large inverted duplications, deletions, amplification, chromosomal translocation have all been observed to be associated with HBV integration. In addition, the regulatory proteins HBx and the PreS2 activators can exert a tumor promoter-like function, resulting in positive selection of cells producing a functional regulatory protein.

HCV CARCINOGENESIS

HCV infection is a more important factor than HBV in the development of HCC in western countries. Markers of HCV infection are found in a higher proportion of HCC patients than that in most of the Asian countries; ranging from 44% to 66% in Italy, 27% to 58% in France, 60% to 75% in Spain^[10]. Japan, unlike other Asian countries, also has a high proportion of HCC caused by HCV infection accounting for 80% to 90% of all the cases^[10].

HCC risk increases to 17-fold in HCV-infected patients compared with HCV-negative subjects^[11]. The risk of HCC occurrence is different among all HCV patients. It is a function of the degree of liver fibrosis and the time of acquisition of the infection. The risk for cirrhotic HCV patients (F4) was the highest with 5.8% per year, compared to those who had less fibrosis (F1-3, 0.5% to 2.6%)^[12].

Because of the absence of reverse transcription activity of the HCV RNA virus, its viral genome unlike HBV is not able to integrate into the genome of the infected cell. Therefore, HCV causes HCC *via* an indirect pathway by causing chronic inflammation, cell death, proliferation and cirrhosis. Accordingly, HCV-related HCCs are almost exclusively found in patients with cirrhosis. However, there are studies raising the possibility that HCV may also operate through other pathways in promoting malignant transformation of hepatocytes. HCCs without cirrhosis in HCV-infected patients, though rare, have been reported. The transforming potential of NS3 protein and core protein has been described.

CLINICAL DIFFERENCES BETWEEN HBV- AND HCV-RELATED HCC

Apart from the difference in the carcinogenic mechanisms, there are a number of clinical differences between HBV- and HCV-related HCC. For reasons yet to be known there is a larger proportion of male HBV infected patients with HCC than the HCV counterpart. The male-to-female ratio is higher in HBV-related HCC than in HCV-related HCC (6.7:1 and 3.3:1 respectively in one study)^[13]. Studies consistently show that HCC develops 10 years earlier in HBV carriers than in HCV carriers, a difference possibly due to the earlier age at exposure to the causative agent^[13,14].

Due to the different mechanisms involved in carcinogenesis, their rate of HCC development is also different. In a Japanese study, 795 patients with viral or alcoholic cirrhosis were observed from 1974 to 1989. Two hundred and twenty five patients (27.8%) developed HCC. The cumulative rates of HCC development in the 180 HBV cirrhotic subgroup at 3, 5, 10 and 15 years after follow up were 7.2%, 14.2%, 27.2% and 27.2% respectively. At 9 years after the follow up, the rate of HCC development began to decrease, and the cumulative rate remained the same thereafter. The corresponding rates in HCV-related cirrhosis were 10.4%, 21.5%, 53.2% and 75.2% respectively in the 349 HCV patients. The authors conclude that the cumulative rate of HCC occurrence has a linear relationship with time in HCV patients whereas it seems to plateau after 10 years for HBV patients^[15,16]. However, according to an Italian study, the cumulative rates of HCC at 5, 10 and 15 years continue to increase with 6.5%, 23.4% and 31.9% for patients with HBV-related cirrhosis, and 4.6%, 24% and 56.2% for patients with HCV-related cirrhosis, respectively^[17]. At the time of HCC diagnosis, a higher proportion of HCV infected patients than HBV infected patients has advanced liver histology and has a higher Child Pugh's score^[13].

The HCC tumor size and the growth pattern also tend

to be different between HBV- and HCV-related HCCs. In most patients with HCV-related HCC, the tumors are more likely to be solitary, smaller sized and encapsulated whereas HBV-related HCC are more commonly infiltrative and multinodular^[18]. Extensive hepatic involvement and portal vein invasion by the tumor were found in 44% and 52% respectively in 95 HBsAg-positive HCC patients compared to 25% and 28% respectively in 370 HBsAg-seronegative HCC patients^[19].

NATURAL HISTORY OF HCC

The natural history of HCC depends on tumour growth characteristics as well as the underlying liver cirrhosis. The majority of hepatitis-related HCC develops in cirrhotic livers with a relatively poor liver reserve. Given a wide range of tumor doubling time, some patients may die from liver failure before the tumor grows to an advanced stage. Features of HCC shown to indicate more aggressive behaviour include poorly differentiated histology, lack of fibrous capsule, large tumour size, early vascular invasion and elevated serum levels of alpha fetoprotein (AFP). Tumor growth rates have a wide range of variability even among patients of the same region and regardless of disease stage. Doubling time ranges from 1 mo to 19 mo with a median of 4 to 6 mo^[20].

Vascular supply of HCC is derived from the hepatic arterial network and the efferent vessels of portal origin have been described to create arteriportal or arteriovenous shunts which serve as a low resistance path for tumour thrombi fragments to survive and spread within the portal efferent network. In a large autopsy study from Sweden, 56% of HCC had evidence of vascular invasion but biliary tract involvement was observed in only 4%^[25]. In another autopsy study in Japan, vascular involvement was noted up to 82% and the likelihood of vascular involvement was related not only to tumor size but also the macroscopic types^[26]. The frequency of portal vein invasion is higher than hepatic vein invasion^[27].

A retrospective analysis was performed in the United States of America on 347 HCC patients who received a metastatic workup including bone scans and computed tomography scans of the chest, abdomen, and pelvis. Clinical and tumor characteristics were evaluated as risk factors for metastasis by univariate and multivariate methods. One hundred forty-five patients had metastases: 72 had thoracic, 57 had abdominal, and 34 had bone metastases. Poor tumour differentiation, multilobular spread, and tumour size of 5 cm or more were the strongest predictors of metastatic disease by logistic regression analysis^[28]. In a Japanese study, the relationship between HCC specimen staining for AFP, serum AFP levels and pathological findings were examined. The disease prognosis was studied with respect to the staining for AFP in excised tumors. The mean serum AFP level in patients with positive AFP staining was significantly higher than in those with negative AFP staining. No significant relationship was found between AFP positivity and tumor size, tumor encapsulation, degree of vascular invasion, or the histological differentiation grade of the tumor. However, patients with AFP-positive

carcinomas had a poorer prognosis than those with AFP-negative carcinomas (5-year survival rate of AFP-positive and negative groups were 26.7% and 56.5%, respectively)^[29].

PREDICTION OF SURVIVAL

The prognosis of HCC has been dismal because patients with HCC usually present late and have already developed advanced liver cirrhosis. Curative resection was seldomly possible. Therefore, the life expectancies of patients with newly diagnosed HCC were classically measured in terms of weeks to months with mortality to incidence ratio close to 1. Due to the increased awareness of the disease and HCC surveillance programmes in some parts of the world, HCC can be diagnosed at its earlier phase permitting timely curative interventions. In a Hong Kong study^[30], the clinical features and disease outcome of 306 HCC patients were analysed. The characteristics of those present with symptoms and those with the disease diagnosed by screening were compared. One hundred and forty two patients with HCC diagnosed by regular screening have a significantly lower serum AFP level, smaller tumour size, less bilobar disease, less portal vein infiltration and less distant metastasis compared with the 164 symptomatic subjects. As a result a higher proportion of patients could undergo curative resection (26.8% *vs* 7.9%).

Scores and models for predicting survival probability are invaluable to tailor suitable treatment options. The traditional TNM cancer staging system has limited applicability as it does not take into account the possible poor liver function in HCC patients. Eighty percent of HCC patients cannot be treated with surgery, so the tumour pathologic information needed in TMN cancer staging is not available. This further limits its prognostic use. Child-Pugh score on the other hand was designed to predict survival in liver cirrhosis without any component of HCC tumour morphology and consideration of disease extension. The first widely accepted staging system to predict survival for HCC which incorporates tumour biology and hepatic function was proposed by Okuda *et al* in 1985^[31]. Tumour size, serum bilirubin, albumin and the presence of ascites are included in the calculation of Okuda staging. In a Hong Kong study of 106 untreated unresectable HCC patients performed in 2001, the median survival was 5.1 mo in stage I, 2.7 mo in stage II and 1.0 mo in stage III. These figures were remarkably similar to the original report by Okuda, in which the median survival of stages I, II and III disease was 8.3, 2, and 0.7 mo respectively. Among other staging systems, only Okuda staging was independently correlated with prognosis in the final multivariate model. As more treatment options become available, newer prognostic models are introduced. The Cancer of the Liver Italian Group Programme (CLIP) proposed a staging system which has been shown to be superior to that of the Okuda stage with a higher number of categories and greater discriminant ability. It reveals a subgroup of patients with an impressively more favourable prognosis who could be candidates for more aggressive therapeutic strategies. On the other hand, it also sorts out a subset of patients with a median survival long enough to be considered for clinical trials of palliative

antineoplastic treatment. It includes Child-Pugh score, tumour size, presence of portal vein thrombosis, and serum AFP. Another survival model is the Barcelona Clinic Liver Group (BCLC) staging system which also effectively selects patients for aggressive treatments. It is constructed from several cohort studies and randomized controlled trials (RCTs) by the Barcelona group. This classification uses variables related to tumour stage, liver functional status, physical status, and cancer-related symptoms, and links the five stages described (0, A-D) with a treatment algorithm. The therapeutic approach configured into the BCLC staging system for patients with non-surgical HCC is based on the study by Llovet *et al* in which asymptomatic patients without evidence of disease extension (vascular invasion or metastasis) had significantly better survival compared to those with more advanced disease^[28]. Such patients are classified as having intermediate HCC and may be better candidates for tumour ablative interventions or chemoembolization.

HBV VACCINATION

HBV vaccination has been shown to be effective to protect against HBV infection. The vaccination is recommended to high risk population groups such as health care workers, patients with immunocompromised states or with chronic liver diseases or with the need for regular blood product transfusion or hemodialysis, and neonates of HBV carrier mothers. Vertical and early horizontal transmission is the major route of HBV transmission in high prevalent areas. The transmission rate was estimated to be as high as 90% in HBeAg positive mothers. The use of HBV vaccine and hepatitis B immunoglobulin (HBIG) can greatly reduce the infection rate of the neonates to 10% to 15%^[33]. The use of vaccination in all newborns can reduce the chance of infection acquired *via* other routes during early childhood and thus preventing the prolonged immunotolerant phase of this unique infection. By eliminating HBV seroprevalence in the population, HCC incidence should be greatly reduced in high prevalence areas. In Taiwan, a universal HBV vaccination programme was carried out in 1984. A study had shown that the HCC incidence in children was decreased since the introduction of the vaccination programme^[34]. In that study, the author showed that the annual incidence of childhood liver cancer was reduced from 0.70 per 100 000 to 0.36 per 100 000 in the children born between 1981 to 1986 and 1990 to 1994 respectively. Therefore a similar decrease in the adult HCC incidence is expected in the future. Likewise, vaccination programmes in high endemic areas like Gambia, intermediate endemic areas like Italy had shown impressive reduction of the HBV seroprevalence. This may transform to a reduction in HCC incidence similar to the Taiwan study. However, the development of an effective vaccine has been hampered by the high genetic variability of HCV.

CONCLUSION

HCC is a complex neoplasm often occurring in a preneoplastic cirrhotic liver, and thus, survival and treatment options depend on variables of both HCC and cirrhosis.

Among chronic viral hepatitis-related HCC, differences in carcinogenesis and clinical presentation exist between HBV and HCV patients. Survival models using tumour size, invasiveness, liver function, patient physical performance status can predict prognosis and guide treatments.

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Hepatocellular carcinoma, human immunodeficiency virus and viral hepatitis in the HAART era

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Abstract

The incidence of hepatocellular carcinoma (HCC) in patients with human immunodeficiency virus (HIV) is rising. HCC in HIV almost invariably occurs in the context of hepatitis C virus (HCV) or hepatitis B virus (HBV) co-infection and, on account of shared modes of transmission, this occurs in more than 33% and 10% of patients with HIV worldwide respectively. It has yet to be clearly established whether HIV directly accelerates HCC pathogenesis or whether the rising incidence is an epiphenomenon of the highly active antiretroviral therapy (HAART) era, wherein the increased longevity of patients with HIV allows long-term complications of viral hepatitis and cirrhosis to develop. Answering this question will have implications for HCC surveillance and the timing of HCV/HBV therapy, which in HIV co-infection presents unique challenges. Once HCC develops, there is growing evidence that HIV co-infection should not preclude conventional therapeutic strategies, including liver transplantation.

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Key words: Hepatocellular carcinoma; Human immunodeficiency virus; hepatitis; Hepatitis B virus; Hepatitis C virus; Co-infection; Incidence; Transplant; Pathogenesis

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INTRODUCTION

With the increased survival of human immunodeficiency virus (HIV) patients with access to highly active antiretroviral therapy (HAART), it is unsurprising that conditions with a typically long latency are now being observed more frequently. Hepatocellular carcinoma (HCC) is such a disease, usually occurring several decades after the initial infection with hepatitis C virus (HCV) or hepatitis B virus (HBV). While few suspect that HIV infection alone is a risk-factor for HCC (indeed, this has been excluded in large retrospective cohort studies^[1]), associated infection with HCV or HBV is common and the significantly increased risk of HCC with viral hepatitis is well-documented. More is now known of the interaction between HIV and HBV and/or HCV over the long-term. Broadly speaking, HIV co-infection seems to accelerate disease progression and reduces the efficacy of anti-HCV and anti-HBV therapy. However, it is unclear whether HIV infection directly increases the likelihood of HCC in viral hepatitis.

Some other key questions are as follows:

What is the true prevalence of HIV and HBV and/or HCV co-infection worldwide?

How should HIV status affect screening and treatment for HCC?

How should HIV infection influence therapy for viral hepatitis?

Answering these questions is essential to planning effective strategies for the prevention, screening and treatment of HCC in HIV-positive patients in the future. Here we review recent attempts to address these challenges.

THE WORLDWIDE AT-RISK POPULATION- WHAT IS THE TRUE PREVALENCE OF HIV AND VIRAL HEPATITIS CO-INFECTION?

HBV and HCV infection have distinct epidemiological and geographical profiles due to different modes of transmission. In broad terms, HCV transmission is predominantly due to intravenous drug use in Western societies and a mix of IV drug use and iatrogenic transmission in developing countries. These modes of transmission also apply to HBV but in endemic areas the primary route is through sexual contact and vertical transmission. HIV shares modes of transfection

Table 1 Examples of internationally diverse prevalences of HBV and HCV co-infection in HIV populations.

Country	Germany, Ockenga <i>et al</i> 1997 ^[45]	Greece, Dimitrakopoulos <i>et al</i> 2000 ^[45]	Thailand, Sungkanuparph <i>et al</i> 2004 ^[45]	TREAT Asia HIV Observational Database (TAHOD) ^[45]	Spain, Gonzalez-Garcia <i>et al</i> 2002 ^[45]	Australia, Lincoln <i>et al</i> 2004 ^[45]	Ivory coast, Rouet <i>et al</i> 2004 ^[45]
<i>n</i>	232	181	529	1498	2820	3309	501
HCV + ve	23%	13.80%	7.80%	10%	-88%	10.70%	1%
HBV + ve	9%	67.40%	8.70%	10%	-5%	4.80%	26%

with both diseases and therefore its geographical and demographic distribution encompasses that of both HBV and HCV. There are many exceptions to this broad rule, however. In the Far East, for example, the HCV burden is predominantly found in the elderly population and probably represents iatrogenic exposure from poorly sterilized medical equipment in the 1920s^[1]. Rates of HIV co-infection are predictably low in this cohort.

Between HIV positive populations in different countries, relative proportions of HCV and HBV co-infection vary widely according to the prevalence of different high-risk behaviours in the populations tested. Examples of these diverse prevalences are given in Table 1.

Most of these studies are disadvantaged by a small sample size (especially in developing countries) or are limited to one particular at-risk group (e.g. haemophiliacs or IV drug users). Thus wide variation is often seen between studies in the same geographical region. For example, in Greece a recent study of 737 HIV infected individuals found a prevalence of 48.1% and 12.1% of HCV and HBV co-infection respectively^[3], a marked contrast to a study only 6 years earlier in the same country (13.8% and 67.4% respectively^[4]).

The recorded prevalence is likely to be inaccurate for other reasons. Around 30% of HCV antibody-positive patients will have undetectable HCV RNA (i.e. have cleared the virus) and yet most studies of prevalence use the antibody test alone. Hepatitis B surface antigen (HBsAg) is almost universally used as a surrogate of HBV co-infection, yet there is growing recognition for a significant subset of patients who are HBsAg-negative but have detectable HBV DNA on quantitative PCR and/or positive anti-HB core antibodies. So-called “occult” hepatitis B infection is frequently detected in patients with HCV infection, HIV and HCC of non-HCV aetiology. The prevalence of occult HBV co-infection in HCV may be as high as 80% in HCV endemic areas such as Japan^[4]. Occult HBV infection is also more common in HIV-positive than HIV negative patients in HBV endemic areas (22.1% *vs* 2.4%)^[5]. This is especially relevant to studies of HCC in HIV infection, since occult HBV infection seems to maintain its oncogenic potential despite lower viral titres. In a recent Italian study, HBV DNA was detected in 68 of 107 cases of HBsAg-negative HCC (63.5%)^[6] and occult HBV infection has been demonstrated in up to 70% of patients with HBsAg-negative HCC in Japan^[7].

It thus seems that larger prevalence studies using RNA and DNA titres are needed to achieve a more accurate estimate of the true burden of viral hepatitis in HIV, particularly in developing countries where the largest reservoirs of infection exist.

HIV CO-INFECTION AND PROGRESSION TO HCC IN VIRAL HEPATITIS

The pathogenesis of HCC in viral hepatitis

Up to 75% of cases of HCC worldwide are thought to be associated with hepatitis B or C^[8]. While the continual inflammation and repair of cirrhosis is an important pathogenic factor in both diseases, the degree of viral replication is more closely related to HCC risk in HBV than in HCV infection. The risk of HCC in active chronic hepatitis B is some 90-fold greater than age-matched healthy controls but only 9-fold greater in inactive carriers of HBV where viral load is low. Also, viral titres seem to correlate directly with the risk of HCC in hepatitis B^[9].

In hepatitis C, the presence of cirrhosis is a prerequisite to the development of HCC, which occurs in between 1%-4% of patients per annum once cirrhosis is established. Indeed, the increased risk of HCC in cirrhotic patients persists in the absence of the virus after successful eradication with IFN therapy^[10].

These differences probably represent the different underlying pathogeneses of HCC in HBV *versus* HCV^[11]. In the former, the random integration of viral DNA into the host chromosomes (an incidental process not necessary for viral replication) leads to secondary chromosomal rearrangement and genomic instability. The HBV DNA product protein HBx (crucial to replication) transactivates genes involved in cell-cycle control (c-jun and c-myc for example). *In vitro* HBx has been shown to disrupt cell-cycle control and inhibit DNA repair and apoptosis all of which have oncogenic potential in their own right^[12].

By contrast, the HCV does not integrate into host cell DNA. Some HCV core proteins do enter the nucleus and modify a variety of signal transduction pathways. Additionally, oxidative cellular injury occurs as a direct result of HCV core protein expression both *in vitro* and *in vivo*^[13]. Although this may participate in the carcinogenic process to a small degree, the principal oncogenic effect of HCV is mediated indirectly through activation of the immune-mediated inflammation and its downstream effects on cell proliferation and apoptosis.

The incidence of HCC in the HIV positive cohort

The 2001 French Mortavic study^[14] was a prospective 1-year cohort study of 25 178 HIV positive patients across 65 national centres comparing the incidence and causes of death to similar cohorts in 1995 and 1997. Deaths from end-stage liver disease (ESLD) rose significantly from 1995 to 2001 (1.5% to 14.3%) and the proportion of those deaths due to HCC also rose 5-fold (4.7% to 25%). Nearly all deaths from HCC were in patients

with HCV co-infection. Deaths from AIDS during this period fell from 91.6% to 48.7% and overall mortality from 8.15% to 1.05%. The authors thus concluded that increased longevity in the HAART era is the principle reason for increased ESLD and HCC in the 2001 cohort. A large retrospective cohort study of US veterans (following 14018 male veterans from 1997 to 2004) has also confirmed a much higher risk of HCC in the HIV population, nearly exclusively in association with HCV (and to a lesser extent HBV) co-infection^[15]. A third retrospective cohort-spanning the pre-HAART and HAART eras-revealed a much higher incidence of HCC in patients with HIV/HCV co-infection than average and the difference was much more marked in the HAART era (only 5 diagnosis of HCC were made in the entire cohort of 11678 patients in the pre-HAART era, *versus* 22 in the HAART era)^[11]. Smaller, retrospective cohort studies in Europe have yielded similar results. One study of 2383 HIV positive patients found a higher-than-expected incidence of HCC (6 cases in total, four of which had HCV co-infection) compared with the population average^[16].

It is noteworthy that studies examining cohorts in the pre-HAART era or in countries where HAART is not readily available, have frequently found the incidence of HCC to be lower or equal to average population rates^[17,18]. The more recent studies, however, proffer convincing evidence that HCC incidence is higher than the population average and rising amongst the HIV-positive population receiving HAART-nearly exclusively in association with HCV or HBV infection- and cite increased longevity as the principal cause of this phenomenon.

However, none of the above studies indicates that there is a higher incidence of HCC in HIV and viral hepatitis co-infection compared to isolated HCV or HBV infection- as one might expect if HIV accelerates the disease progression of viral hepatitis towards HCC.

HCC oncogenesis in viral hepatitis-is HIV a true additional risk factor?

While the HIV virus itself is not considered to be particularly oncogenic in its own right, a number of cancers are well known to occur with increased frequency in people with HIV infection. For the most part this is a consequence of impaired immunity and failure to clear several common oncogenic viruses such as HHV8 in Kaposi sarcoma (KS), EBV in non-Hodgkin's (NHL) and HPV in anal and cervical cancer.

In vivo studies in murine models have revealed a potential role for the HIV Tat gene in liver tumorigenesis. Transgenic mice expressing this gene have a greater incidence of hepatocellular carcinoma. This is thought to be mediated by extra-hepatic growth signals rather than by direct disruption of the hepatocyte cell cycle by the Tat product and the effect is not specific for hepatocarcinoma; these animals suffer a higher incidence of other extra-hepatic tumours (leiomyosarcomas, squamous cell papillomas and carcinomas, adenocarcinomas of skin adnexa and B-cell lymphomas). In humans, however, large retrospective cohort studies in the HAART era have shown no increased incidence of HCC in HIV monoinfection^[1].

There is clearer evidence that HIV can accelerate disease progression of both HBV and HCV and thus indirectly increase the chance of subsequent HCC. HIV affects the natural history of HCV infection in two important ways: firstly, it increases the likelihood of chronic infection following the acute episode^[19] and secondly, it hastens the development of cirrhosis once chronic infection is established^[20,21]. This has important implications for the subsequent development of HCC and any screening strategy. HIV co-infection has also been shown to accelerate the progression of HBV infection^[22], with patients suffering from more severe disease at an earlier stage. Increased liver injury in viral hepatitis may also be mediated indirectly in HIV by antiretroviral therapy-related hepatotoxicity and by immune reconstitution syndrome. Indeed, HCV infection seems to increase the risk of HAART-related hepatotoxicity^[23]. It must also be considered that once HCC has developed, a putatively weaker anti-tumour response due to chronically low CD4+ and CD8+ lymphocyte counts may result in more rapid growth and spread of disease.

Despite evidence for acceleration of cirrhosis in viral hepatitis with HIV co-infection, attempts to demonstrate a specific increase in HCC in this context-over and above that observed in HCV or HBV monoinfection- have so far yielded variable results.

In the US veterans studies mentioned above, direct comparisons were made between HCV mono-infected subjects and groups with HIV/HCV co-infection.

Mcguinnis *et al* (2001)^[15] compared the incidence of HCC between 14018 HIV positive and 28036 age-, sex- and location-matched HIV-negative controls in a large retrospective cohort study from 1997 to 2004. A higher age-matched incidence of HCC was clearly demonstrated in the HIV positive group (incidence rate ratio 1.68) but when adjusted for HCV infection and/or alcohol consumption the incidence rate ratios were similar, suggesting HIV co-infection confers no additional risk of HCC compared to HCV infection alone.

A precursor of this study (another retrospective cohort of US veterans) compared the incidence of cirrhosis and HCC in 26641 HCV-only with 4761 HCV/HIV co-infected subjects between 1991 and 2000^[24]. The incidence of HCC in both groups was found to be the same in the HAART era and lower in the HIV/HCV co-infection group in the pre-HAART era. This would corroborate the premise that HIV patients did not survive sufficiently long in the pre-HAART era to develop HCC, but also suggests that in the HAART era HIV status does not seem to alter the likelihood of progression to HCC in HCV infection.

These studies, however, are subject to several sources of error. In one retrospective cohort the authors concede that up to 50% of the apparent HCV-only group were never tested for HIV (which would bias the study towards the null) and it was also subject to changes in disease reporting and coding during the study period which may have lead to significant acquisition bias. More importantly, a recurring confounding factor is the rising incidence of cirrhosis (and therefore HCC) throughout the study period in patients with isolated HCV infection. This may reflect earlier acquisition of HCV in this group (often in

the 1970s in the US) compared to their HIV co-infected counterparts and highlights a recurring deficiency in such retrospective cohort studies: they rarely include data on when infection was acquired. Although HCC rates may be similar in HIV/HCV and HCV-only groups in the immediate post-HAART era, the HIV/HCV co-infected cohort may well have acquired HCV more recently and thus the equal incidence may actually belie accelerated disease progression in this group.

The 2004 Italian cooperative group on AIDS and Tumours (GICAT) study examined 41 cases of HCC in HIV positive individuals (from a joint Italian and Spanish database) and compared them with 384 HIV-negative controls diagnosed over the same period (1995-1998)^[25]. This is the largest study purporting to show an acceleration of liver disease towards HCC in HIV and viral hepatitis co-infection. The HIV group with HCC were much younger at presentation (age 40-46 *vs* 60-70) and had more advanced infiltrating disease. There was also a trend to more advanced cirrhosis at presentation in the HIV positive population. Accordingly, few of the HIV patients were offered active treatment and survival rates were poor. Again, HCV co-infection was clearly the main risk factor in both the HIV positive group and negative controls. In this study, the younger age at diagnosis and the limited data available on the timing of HCV infection in both HIV co-infected and HCV-only groups suggested HIV-HCV co-infected patients develop HCC some 10 years earlier than expected (compared with a previous series examining HCC in HIV-negative patients with post-transfusion HCV).

Three further studies have also described earlier development of HCC (and poorer outcome) in HIV co-infected subjects compared with HBV or HCV monoinfection, including one cohort of British haemophilic men and boys^[26], one of homosexual men in the United States^[27] and a Spanish retrospective cohort of 2383 HIV positive subjects^[28]. The total numbers of cases of HCC in each study were very low, however.

Convincing evidence for an HIV-induced acceleration of disease progression in viral hepatitis towards HCC thus remains lacking. Of note is a glaring lack of studies specifically addressing HBV and HIV co-infection—presumably because this is far less prevalent than HCV/HIV co-infection in the developed countries which have the advanced information infrastructure needed to carry out retrospective trawls of large databases.

If one postulates the ideal study to address whether HIV is a true additional risk factor for HCC in viral hepatitis, it would consist of a prospective cohort of HIV/HCV/HBV co-infected and HCV- and/or HBV-only subjects with all subjects cross-tested for co-infection before allocation to groups (and at regular intervals thereafter). It would also instigate screening for HCC on a regular basis. It might be reasonable to expect that even if HIV has an accelerative effect on HCC pathogenesis this might be countered viral suppression by HAART, therefore such a study would undertake regular monitoring of HIV viral load in the HIV co-infected group. Crucially, if the duration of HIV and HBV/HCV infection is known for each patient, fewer patients and years of follow-up would be necessary to detect an accelerative affect of HIV

on progression to HCC.

It is telling that almost none of the studies mentioned above include any such parameters. On the basis of existing evidence, we can only conclude that if an additional risk of progression to HCC in viral hepatitis is conferred by HIV it is not large enough to be detected by relatively crude retrospective examination within the short space of time (relative to normal HCC pathogenesis) that has passed since the introduction of HAART.

SCREENING, PREVENTION AND TREATMENT OF HCC IN HIV

Treatment and outcome of HIV patients with HCC

In the HIV negative population, solitary or a small number of HCC lesions are resectable, and associated with a 5-year survival of 60%-70%^[27]. In the presence of cirrhosis patients with operable lesions are offered transplantation resulting in equivalent survival data. Operability is determined by the Milan criteria (no evidence of extrahepatic tumour and unifocal tumour mass < 5 cm in diameter or multifocal tumours < 4 in number, each < 3 cm in diameter^[29]), although some large-volume centres now adopt the more aggressive University of California, San Francisco (UCSF) criteria which have extended tumour burden limits with similar outcomes^[30]. Ethanol injection is another treatment option for patients with local disease who are not candidates for surgery and is associated with 5-year survival rates of approximately 50%^[31]. Patients with more advanced disease are limited to palliative embolization. No chemotherapy or targeted therapy has been shown to offer a survival benefit for these patients.

Data from other HIV positive non-AIDS defining cancers, such as lung cancer, suggest these patients are offered curative therapy less frequently than their HIV negative counterparts due to the advanced nature of their disease at presentation^[32]. In the series described in the GICAT study^[32] 15 of the 41 patients with HIV and HCC had disease within the Milan criteria that would be deemed curable with liver transplantation. However, none underwent liver transplantation and only two underwent surgical resection. Overall, HIV positive patients have a much worse outcome compared to their HIV negative counterparts with only a 28% 1 year survival.

Although HIV-positive status was previously an absolute contraindication to liver transplantation, this is now becoming more commonplace following several transplant series which have demonstrated similar survival outcomes in MELD-score matched HIV- positive and negative recipients^[33-38]. There is no evidence, as previously feared, that the subsequent immunosuppressive therapy results in progression of HIV disease^[39].

There is now data specifically addressing liver transplants in HIV positive patients with HCC. Di Benedetto *et al* recently reported a series of 7 patients with HIV and HCC who fulfilled Milan criteria and underwent liver transplantation^[40]. After a mean follow-up of 232 d, the overall patient and graft survival was 85.7%. One patient died of a myocardial infarction with a functioning graft and no evidence of HCC recurrence.

Liver transplant patients with HIV, for any indication, clearly face specific problems post-transplant compared with HIV negative counterparts. These include more aggressive HCV re-infection, more common lamivudine resistance in HBV and a higher incidence of tacrolimus toxicity^[41]. However, where an increased mortality has been observed it is frequently cited that late referral plays a role^[42]. A greater proportion of HIV-positive patients seem to die on the waiting list for liver transplantation from liver-related causes. Two conclusions can therefore be drawn: firstly, patients with HIV and HCC should not be denied the opportunity for liver transplantation and secondly, detecting these cancers early is of paramount importance.

Preventative therapy in HIV and HBV/HCV co-infected patients

Patients with HIV-HCV co-infection benefit from HCV eradication for the same reasons as those with HCV alone. Sustained virological response rates (SVR) of between 27%-40% have been achieved with IFN and ribavirin therapy in HIV co-infected patients^[43]. Not only is the risk of HCC reduced with HCV eradication but the resulting enhanced liver function increases tolerance to antiretroviral agents. Treatment of the HIV-HCV patient presents several challenges however, such as (1) more severe and frequent IFN related myelosuppression, (2) more frequent haemolytic anaemias caused by ribavirin interaction with zidovudine, (3) antagonism of zidovudine phosphorylation (4) increased risk of lactic acidosis. Most significantly, patients with low CD4+ counts respond very poorly to HCV eradication therapy^[44].

In HIV-HBV infection, there is every reason to suspect the risk of progression to HCC can be reduced by viral suppression just as it is in monoinfected HBV cases. Given that HAART and HBV suppression regimens share many common agents (lamivudine, for example) the principal problem encountered in this group is cross-resistance. Dual therapy for effective HBV suppression is frequently required (e.g. lamivudine plus tenofovir) but, as in HCV coinfection, response rates are poor in patients with low CD4+ counts.

Although anti-viral therapy in HIV and HBV and/or HCV is more complex and generates more potential adverse events, if early hepatological expertise is sought then the vast majority of patients should have the option of potentially curative therapy in HCV and effective long-term viral suppression in HBV.

Screening for HCC in patients with hepatitis and HIV co-infection

The European association for study of the liver (EASL) has released guidelines for screening HIV and HCV/HBV co-infected individuals. These are similar to HCV and HBV monoinfected patients with established cirrhosis and recommend screening every 6 mo with ultrasonography and alpha-fetoprotein levels. In the United States suggested screening of co-infected individuals incorporates the use of ultrasound as the primary screening modality. They suggest AFP should not be used alone unless

ultrasonography is unavailable; screening should occur at 6-12 mo intervals and should include all those at elevated risk for HCC^[32,38].

In our unit we currently do screen co-infected individuals with both α -fetoprotein (AFP) and ultrasound scans if the AFP is raised. This has not been validated and early unpublished results suggest these may be problems with sensitivity and specificity of AFP in this setting, especially as HAART can cause an increase in AFP levels^[41]. Anecdotally our programme has revealed a low sensitivity and specificity. This may in part be due to patient selection and the potentially rapid development of the disease. More frequent imaging surveillance in very high-risk groups (such as those with persistently high HBV titres, for example) may prove to be the way forward. Data in this setting is urgently required.

CONCLUSION

Retrospective cohort studies warn us that an increase in HCC in the HIV population is already underway in regions where HAART therapy is available. Increased access to HAART can be expected to have similar consequences in developing countries but the timing of this increase will be difficult to predict. Insufficient epidemiological data on HIV and HBV/HCV co-infection (especially in the developing countries which are most affected) makes the at-risk population very difficult to locate and quantify. Because the latency of HCC in HIV co-infection may be shorter than in isolated viral hepatitis, there may be less time than we might expect to prepare adequate screening, prevention and treatment services.

Such services are currently far from optimal. A recent US study of HIV clinic management of HBV co-infection found that HBV viral load was inadequately monitored in HIV co-infected patients despite regular measurements of HIV titres^[45]. Other guidelines for HBV management were loosely adhered to. The applicability of current HCC screening techniques in viral hepatitis mono-infection remains untested in the context of HIV co-infection, and once HCC is diagnosed, patients are referred for potentially effective treatment less frequently and much later than their HIV-negative counterparts.

Although guidelines now exist on the management of viral hepatitis and HIV co-infection, there will always be unique scenarios not covered by these that present both opportunities (e.g. anti-viral cross-efficacy) and pitfalls (e.g. cross-resistance). As the HAART era takes us into uncharted waters, holding to the optimum course through prevention, screening and therapy for HCC will require both HIV physician and hepatologist at the helm.

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TOPIC HIGHLIGHT

Harry HX Xia, PhD, MD, *Series Editor*

Radioembolization for the treatment of unresectable hepatocellular carcinoma: A clinical review

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Abstract

Hepatocellular carcinoma (HCC) is the sixth most common cancer in the world. The majority of patients with HCC present with unresectable disease. These patients have historically had limited treatment options secondary to HCC demonstrating chemoresistance to the currently available systemic therapies. Additionally, normal liver parenchyma has shown intolerance to tumoricidal radiation doses, limiting the use of external beam radiation. Because of these limitations, novel percutaneous liver-directed therapies have emerged. The targeted infusion of radioactive microspheres (radioembolization) represents one such therapy. Radioembolization is a minimally invasive transcatheter therapy through which radioactive microspheres are infused into the hepatic arteries that supply tumor. Once infused, these microspheres traverse the hepatic vascular plexus and selectively implant within the tumor arterioles. Embedded within the arterioles, the ^{90}Y impregnated microspheres emit high energy and low penetrating radiation doses selectively to the tumor. Radioembolization has recently shown promise for the treatment of patients with unresectable HCC. The objective of this review article is to highlight two

currently available radioembolic devices (^{90}Y , ^{188}Rh) and provide the reader with a recent review of the literature.

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Key words: Radioembolization; Brachytherapy; Hepatocellular carcinoma; Yttrium-90; Rhenium-188

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INTRODUCTION

Hepatocellular carcinoma (HCC) claims half a million lives across the globe each year^[1]. It is the sixth most common cancer in the world and is the third most common cause of cancer-related mortality^[2]. Various etiologic factors have been implicated in the transformation of benign hepatic parenchyma to malignancy, however, no one factor has been shown to cause cancer in all cases. Although several postulates for tumorigenesis have been proposed, the exact underlying mechanism for neoplastic change remains unknown^[1,3].

The incidence of HCC varies considerably across geographic regions with some areas reporting cases as high as 20/100 000 per annum^[3]. Various studies have shown that advanced age and male sex portends a higher likelihood of developing HCC. Several important risk factors have been identified which substantially increase the possibility of developing disease. Among these, the most common risk factor recognized worldwide is the hepatitis B carrier state. Others inciting factors include chronic hepatitis C infection, cirrhosis, environmental toxins such as aflatoxin and contaminated drinking water, alcohol abuse, diabetes mellitus, and hereditary hemochromatosis.

In patients diagnosed with this lethal malignancy, less than 15% are candidates for surgical procedures. A survival

benefit has been observed in patients that meet the rigorous criteria for curative resection or transplantation^[4]. For the remaining majority, various treatment options have become available without universal agreement on which treatment option offers the greatest survival benefit with the least toxicity.

The use of external beam irradiation has historically played a limited role in the treatment of HCC due to the radiosensitive nature of normal hepatic tissue^[5]. Investigators have shown that liver exposure to radiation doses greater than 40 Gy may result in a clinical syndrome characterized by ascites, anicteric hepatomegaly, and elevated liver enzymes weeks to months following therapy^[5,6]. Additionally, a condition recognized as veno-occlusive disease, marked by central venous congestion and atrophy of adjacent hepatocytes, may develop. Together, the clinical and pathologic spectrum described above has been referred to as radiation induced liver disease (RILD) or radiation hepatitis. This is the most prominent treatment related complication in patients undergoing hepatic irradiation from external sources^[7].

Given this limitation and the need for higher doses to inflict lethal injury to malignant tissue^[8-10], minimally invasive intra-arterial devices have emerged. These devices, loaded with radioactive Yttrium-90 microspheres or Rhenium-188, can deliver very high tumoricidal doses without the development of RILD^[11]. Using segmental infusion techniques, doses as high as 4993 Gy to liver tissue have been reported^[12]. Although the use of these devices dates back to the early 1960's, only recently has the therapeutic safety and efficacy associated with its use been realized^[13,14]. For the purpose of this review, we aim to highlight the use of intra-arterial radiotherapy for the treatment of inoperable HCC and update the reader on recent clinical and research advancements.

DEVICE AND DOSIMETRY CONSIDERATIONS

Yttrium-90 intra-arterial radiotherapy, also known as radioembolization, is a minimally invasive catheter-based therapy that delivers internal radiation via the arterial vessels that feed tumors. "Radio" refers to the radiation that is imparted to tissue; "embolization" refers to the microembolic effect^[15]. This technology takes advantage of the dual blood supply to the liver. Normal hepatic tissue derives greater than 70% of its blood supply by way of the portal system whereas malignant tissue is preferentially supplied by the arterial system. There are currently two commercially available Yttrium-90 microsphere devices. TheraSphere[®] (MDS Nordion, Ottawa, Ontario, Canada) is made of glass and SIR-Spheres[®] (Sirtex Medical, Sydney, Australia) is made of resin. These two devices are different in a number of important respects^[16]. TheraSphere[®] is a minimally embolic device consisting of 20-30 micron particles with higher specific activity (2500 Bq) and lower number of spheres (1.2 million microspheres/3 GBq). Conversely, SIR-Spheres[®] are moderately embolic, consisting of 20-60 micron particles, with lower specific activity (50 Bq), and greater

number of spheres (approximately 40-80 million spheres/3 GBq). A third agent, available in certain countries, uses a Rhenium-188 based radioconjugate delivered in a trans-arterial manner analogous to the Yttrium-90 based devices^[14].

^{90}Y Glass microspheres

^{90}Y microspheres (TheraSphere[®], MDS Nordion, Ottawa, Canada) are composed of nonbiodegradable glass microspheres ranging from 20 to 30 μm in diameter, in which ^{90}Y is an integral constituent of the glass. ^{90}Y is a pure β -emitter with a physical half-life of 64.2 h, after which ^{90}Y decays into stable zirconium. The average energy of β -emission is 0.9367 MeV, mean tissue penetration of 2.5 mm and a maximum penetration of 10 mm. One gigabecquerel (27 mCi) of ^{90}Y per kilogram of tissue provides a dose of 50 Gy. The microspheres are supplied in 0.5 mL of sterile, pyrogen-free water contained in a 0.3-mL V-bottom vial secured within a 12-mm clear acrylic shield. The specific activity is 2500 Bq at the time of calibration.

The typical method of calculating the required activity level (in GBq) to be injected and the actual dose delivered to the liver and lung has been previously published. CT or MR imaging is used to determine the targeted liver volume to be treated with ^{90}Y microspheres^[17-19]. The targeted liver volume is that portion of liver tissue that will be perfused once the catheter is in the desired location. A conversion factor of 1.03 g/cm³ is used to calculate the corresponding targeted liver mass from the targeted liver volume. The required activity is calculated from the following formula:

Activity (GBq) = [target dose (Gy) \times target liver mass (kg)]/50

When lung shunt fraction (LSF) and percentage of residual activity (R) in the vial after treatment are taken into account, the actual dose delivered to the target mass is calculated by rearranging the previous equation as follows:

Dose (Gy) = [Infused activity (GBq) \times 50 \times (1- LSF) \times (1-R)]/liver mass (kg)

Cumulative liver dose is defined as the accumulated dose to that specific volume that was treated multiple times. By targeting delivery to a hepatic segment or lobe, ^{90}Y therapy results in high radiation doses to the tumor while sparing liver parenchyma. These tumoricidal doses have proven effective in the ability of ^{90}Y microspheres to reduce tumor viability, demonstrating an increasing therapeutic effect with radiation dose^[11].

^{90}Y Resin microspheres

SIR-Spheres[®] consist of biodegradable resin-based microspheres containing ^{90}Y . The average size of a sphere is 35 microns in diameter. Upon *in vivo* administration, the spheres are permanently implanted. Each vial contains 3 GBq of ^{90}Y in a 5 mL vial. Each vial contains 40-80 million spheres. The activity per microsphere is 50 Bq at the time of calibration.

The radioactivity to the liver can be calculated by one of two methods:

(1) The first method allows the calculation be based on body surface area to determine an approximate tumor burden:

Activity (GBq) = body surface area (m²) - 0.2 + (% tumor burden/100)

(2) Based on a broad estimate of tumor burden which then requires the user to increase the recommended activity by 0.5 GBq per 25% increase in tumor burden.

Activity for tumor involvement < 25%, 25%-50% and > 50% are 2.0 GBq, 2.5 GBq and 3.0 GBq, respectively.

Using either dosimetry model, activity administered is decreased depending on the extent of identified lung shunt. Also, recent clinical practices have shown that an additional 25%-30% activity reduction is usually necessary for SIR-Spheres[®][20]. The dosimetry model for SIR-Spheres[®] is based on whole liver infusion. If a lobar administration is intended, the activity to be administered should be calculated using whole liver volume and then corrected for the target volume anticipated for treatment. As an example, if a right lobe infusion is anticipated, the calculated GBq should be multiplied by the percentage of right lobe as a proportion to the entire liver. Dosimetric issues and technical considerations have been described in detail previously^[16,20,21].

Rhenium-188 radioconjugate

This is available through the use of a Rhenium-188 generator. The half-life of Rhenium-188 is 16.9 h. The isotope delivers high-energy beta (2.1 MeV max) and a low energy gamma (155 keV) emissions, permitting imaging. Usually, this radioconjugate is in the form of Rhenium-188 4-hexadecyl 1, 2, 9, 9-tetramethyl-4, 7-diaza-1, 10-decaethanol labeled with iodized oil. Dosimetry is based on the safe and tolerable dose to organs at risk including the liver, lungs and bone. A small scout dose of the radioconjugate (3.7 MBq) is administered on the day prior to treatment. Subsequently, transmission scans with a Rhenium-188 flood source are performed to determine the attenuation correction factors for lung and liver to be used in the dosimetric calculations the following day. Anterior and posterior images are obtained to calculate geometric mean counts. After correcting for scatter, regions of interest are placed around the whole liver, tumor and lungs. Using medical internal radiation dosimetry and by adjusting for the difference in total body and organ masses between the patient and the anthropomorphic model, the proper activity required is calculated using the following dose limitations: 12 Gy to the lungs, 30 Gy to the normal liver, 1.5 Gy to the bone marrow.

Absolute and relative contraindications

Two absolute contraindications exist for the use of ⁹⁰Y microsphere treatment in any patient. The first includes a pretreatment ^{99m}Tc macro-aggregated albumin (MAA) scan demonstrating significant hepatopulmonary shunting that would result in > 30 Gy being delivered to the lungs with a single infusion or as much as 50 Gy for multiple infusions. The second includes the inability to prevent deposition of microspheres to the gastrointestinal tract with modern catheter techniques. A number of relative contraindications exist including non-compromised pulmonary function, adequate liver reserve, serum creatinine < 2.0 mg/dL, and a platelet count > 75 × 10⁹/L. For relative contraindications, clinical judgment should be exercised when determining whether a patient is appropriate to undergo this procedure.

Observed toxicities

The most common clinical toxicity observed with the use of ⁹⁰Y is a mild post-embolic syndrome. This syndrome, unlike that observed with other embolic treatments such as transarterial chemoembolization (TACE), includes fatigue, vague abdominal discomfort, pain, and fever^[11,22,23]. Other avoidable toxicities that occur as a result of non-target radiation include: cholecystitis, gastric ulceration, gastroduodenitis, pancreatitis, radiation pneumonitis, and RILD^[16,24-27]. With meticulous planning, careful selection, and proper technique, the majority of these toxicities can be mitigated. Finally, hematologic toxicities seen in the immediate post-procedural period include lymphopenia. This is not an unexpected finding given the sensitivity of lymphocytes to radiation. Despite this, no infectious complications have been documented^[16,21,28].

LITERATURE REVIEW

A comprehensive literature review was completed in 2006 describing the entire clinical and scientific evidence for ⁹⁰Y in detail^[13]. Since then, additional evidence has been generated^[16]. A consensus panel report from the radioembolization brachytherapy oncology consortium concluded that there is sufficient evidence to support the safe and effective use of this loco-regional therapy in HCC patients^[20]. The authors further suggested the need to investigate the benefits of ⁹⁰Y in combination with other traditional therapies. The results from phase I and II studies in combining ⁹⁰Y with targeted therapies (Raf-kinase, EGFR) for HCC are underway and should provide valuable insight into the toxicity and efficacy of such regimens.

Sangro *et al* reported on 24 HCC patients with Child-Pugh A disease who underwent radioembolization with resin microspheres^[29]. The median activity delivered was 2.2 GBq. The investigators reported a reduction in size of target lesions in 19 patients. Using RECIST criteria, 88% of the cohort had either partial response or stable disease. The authors did not observe any post-embolization syndrome and all patients were discharged within 24 h of treatment. Two patients became jaundiced at 1 mo and 3 mo after the procedure from uncertain causes. Two treatment-related deaths were recorded. At median follow-up of 12.5 mo none of the treated patients progressed. Given the tumor response and minimal toxicity profile, the investigators concluded that radioembolization is a viable therapy for patients with portal vein thrombosis and preserved liver function and that this therapy needs to be considered in patients who are awaiting transplant in order to prevent extension of disease beyond the Milan criteria.

Rivera *et al* presented a case report of a 42 years old hepatitis C cirrhotic male with tumor recurrence 22 mo post-transplantation^[30]. The investigators in the study then treated the patient with ⁹⁰Y resin microspheres. The author noted no change in liver function post-procedurally and follow-up MRI demonstrated the absence of arterial enhancement and tumor necrosis. The authors concluded the use of ⁹⁰Y for post-transplant recurrence may help prolong patient and graft survival in patients that develop recurrence.

Gulec *et al* retrospectively analyzed the data from a heterogeneous cohort of 40 patients with primary and

metastatic liver malignancies who underwent single whole liver treatments using ^{90}Y resin microspheres^[31]. The average administered activity was 1.2 GBq and tumor absorbed doses ranged from 40.1 to 494.8 Gy. Sixty-seven percent of the treated cohort responded to therapy with favorable responses reported in those with higher tumor flow ratios. The authors concluded that doses up to 100 Gy to the uninvolved liver were tolerated by this procedure without the development of veno-occlusive disease or liver failure. The authors further noted that lowest tumor dose necessary to generate a detectable response was 40 Gy.

Kamel *et al* reported on 13 patients prospectively treated with ^{90}Y glass microspheres. MR imaging was compared 24 h pre-treatment to an average follow-up of 55 d post-therapy^[32]. Targeted tumors demonstrated a mean decrease in arterial enhancement of 22%, a mean decrease in venous enhancement of 25% and unchanged tumor size in both targeted and non-targeted tumors. The authors reported a median survival of 12 mo from time of diagnosis and emphasized the need for surrogate imaging measures such as diffusion-weighted MR in order to assess response.

Keppke *et al* reported on the imaging findings and median survival of 42 patients using ^{90}Y glass microspheres^[33]. The response rates according to WHO, RECIST, necrosis and combined criteria (RECIST and necrosis) were 26%, 23%, 57% and 59%, respectively. The median survival for Okuda I patients was 660 d. The authors concluded that the imaging findings, using a combined criteria (size and necrosis), resulted in a more accurate assessment of tumor response after ^{90}Y radiotherapy when compared to size criteria alone.

In an attempt to address the question of retreatment using this therapy, Young *et al* recently reported on the relationship between cumulative radiation dose and the development of liver toxicities in 41 patients stratified to Okuda I and II^[34]. The authors observed a statistically significant mean cumulative radiation dose of 390 Gy and 196 Gy tolerated by Okuda I and Okuda II patients, respectively, before the occurrences of toxicity. This suggests that some patients can tolerate multiple treatments prior to the development of liver toxicities. Median survival from date of first treatment for Okuda I and Okuda II were 660 d and 431 d, respectively ($P = 0.44$).

More recently, Kulik *et al* reported on 21 patients from a large database of 251 patients who had undergone ^{90}Y glass microsphere therapy and subsequently bridged to transplantation^[35]. Target tumor dose administered was 120 Gy with toxicities including fatigue in the majority of patients (42%). The authors reported a mean reduction in alpha fetoprotein (AFP) of 33% from pre-treatment levels. The investigators noted complete necrosis by pathologic exam in 14 of 21 patients (66%). Four of 21 patients had disease recurrence with a mean time to recurrence of 250 d, a finding not uncommon following transplantation^[36-38]. The authors concluded that treatment with ^{90}Y achieves complete necrosis in the majority of targeted lesions in patients bridged to transplantation, but that recurrence was a possibility despite the radiographic findings of complete necrosis.

Additionally, Kulik *et al* reported on the safety of ^{90}Y in a 108 patient cohort treated with glass microspheres, with

subset analysis comparing patients with and without portal vein thrombosis^[39]. Thirty-seven of 108 patients presented with imaging proven portal vein thrombosis (PVT). Patients were stratified by Okuda, Child Pugh, baseline bilirubin, ECOG, presence of cirrhosis and location of PVT (none, branch, and main). The cumulative dose administered to those with and without PVT were 139.7 Gy and 131.9 Gy, respectively. Liver related adverse events reported included elevation of bilirubin in 40%, ascites in 18%, and hepatic encephalopathy in 4% of the patients with cirrhosis and main PVT. In the patients without cirrhosis, elevated bilirubin occurred in 4%, ascites in 4% and no cases of encephalopathy. Tumor response using WHO criteria and EASL recommendations were 42.2 and 70%, respectively^[40]. Median survival from the date of first treatment for patients without PVT and cirrhosis was 813 d. In patients with branch PVT, survival was 304 d from time of treatment (cirrhotics: 261 d, non-cirrhotics: 427 d). The authors concluded that the microembolic effect of ^{90}Y microspheres did not increase the risk of liver adverse events in patients with proven PVT. Glass microspheres did not result in a microembolic effect that is seen with other loco-regional therapies using larger diameter particles.

Investigators also studied the use of Rhenium-188 for patients with inoperable HCC^[14]. A multicenter clinical trial was completed looking at Rhenium-188 (Rh-188) lipiodol delivered in a transarterial manner. After complete clinical evaluation (including serum alpha-fetoprotein (AFP), tumor burden, patency of portal vein, Child-Pugh and Okuda classification), radiation absorbed dose (rad) to various organs, including tumor, was calculated after injecting 185 MBq of Rh-188 iodized oil *via* the hepatic artery. From this value, the maximum tolerable activity, defined as the amount of radioactivity delivering no more than 12 Gy of rad to lungs, 30 Gy to normal liver, or 1.5 Gy to bone marrow, was calculated and injected. Ninety-three patients were successfully treated with a mean age of 53 years (80 men and 13 women). Mean cumulative dose was 7.8 GBq. Sixty-eight percent of patients had serologic evidence of hepatitis B and/or C; 40% had clinical/radiologic evidence of cirrhosis. Mean tumor diameter was 10.3 ± 4.4 cm, with 40% of patients having more than three lesions; in 50% of patients, tumor was either unilateral, occupying 50% or more of the liver, or bilateral. AFP was elevated in 68% of patients and serum levels exceeding 300 ng/mL was observed in 44% of these patients. There was portal vein thrombosis in 38% of patients, Child-Pugh B disease in 37% of patients, and Okuda stage II or III disease in 50% of patients. Mean first administered activity was $5.3 \text{ GBq} \pm 1.6$, which delivered 88 Gy to the tumor. Treatment was tolerated well. Five patients had complete tumor response, while 17 had a partial response ($> 50\%$ tumor reduction) for an overall objective response rate of 33%. Thirty-five percent of patients had stable disease. Only dose to the tumor was found to be significantly ($P = 0.001$) associated with tumor and/or AFP response. Median survival for the entire cohort was 356 d and varied accordingly with baseline characteristics. Responders by imaging survived longer than those that did not exhibit a response and interestingly was correlated with dose administered to tumor. The

authors concluded that this was a safe, effective, and promising therapy in patients with HCC with favorable cost-benefit profile.

CONCLUSION

Clinical investigations into the use of ^{90}Y radioembolization for the palliative treatment of unresectable hepatocellular carcinoma appear promising. This therapy potentially offers survival benefit with a low toxicity profile, making it an attractive tool in the battle against a uniformly fatal disease. Unlike external beam therapy, radioembolization can deliver high cumulative radiation doses to targeted hepatic segments without the clinical manifestation of RILD. Additionally, investigators have shown favorable survival outcomes in patients with limited hepatic reserve and portal vein thrombosis. These patients were previously excluded from most therapeutic options. Furthermore, this therapy has successfully been used to bridge and downstage patients to resection, ablation or transplantation^[29,41-44]. Although phase II paradigms have provided useful data, there is a need to carry out randomized controlled trials comparing ^{90}Y therapy to those accepted as standard of care for this patient population. These studies will then establish the role of radioembolization within the framework of other universally accepted first line therapies for inoperable disease. Finally, the development of targeted therapies at the molecular level represents the beginning of a new era in the treatment of HCC^[45]. Clinical investigations into combining the cytotoxic effect of ^{90}Y with the cytostatic mechanism of targeted therapies are currently in progress and will provide valuable safety and toxicity data that may translate into improved clinical outcome and overall survival.

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TOPIC HIGHLIGHT

Harry HX Xia, PhD, MD, *Series Editor*

Harnessing the RNA interference pathway to advance treatment and prevention of hepatocellular carcinoma

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Abstract

Primary liver cancer is the fifth most common malignancy in the world and is a leading cause of cancer-related mortality. Available treatment for hepatocellular carcinoma (HCC), the commonest primary liver cancer, is rarely curative and there is a need to develop therapy that is more effective. Specific and powerful gene silencing that can be achieved by activating RNA interference (RNAi) has generated enthusiasm for exploiting this pathway for HCC therapy. Many studies have been carried out with the aim of silencing HCC-related cellular oncogenes or the hepatocarcinogenic hepatitis B virus (HBV) and hepatitis C virus (HCV). Proof of principle studies have demonstrated promising results, and an early clinical trial assessing RNAi-based HBV therapy is currently in progress. Although the data augur well, there are several significant hurdles that need to be overcome before the goal of RNAi-based therapy for HCC is realized. Particularly important are the efficient and safe delivery of RNAi effectors to target malignant tissue and the limitation of unintended harmful non-specific effects.

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Key words: RNA interference; Hepatocellular carcinoma; Hepatitis B virus; Hepatitis C virus; Molecular pathogenesis; Delivery vectors

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INTRODUCTION

Globally, hepatocellular carcinoma (HCC) is a major cause of mortality^[1,2]. It is the commonest primary liver cancer and accounts for 80%-90% of this class of malignancy. HCC is characterized by a very poor prognosis and is associated with high mortality. Annual mortality from HCC is virtually the same as its annual incidence, attesting to its rapid course and grave prognosis. Moreover, although HCC is the fifth most common cancer worldwide, it is the third most common cause of cancer-related mortality^[3]. Recent observations have shown that incidence and mortality from liver cancer in the United States is the fastest growing of all cancers^[2]. This is despite a decline in the overall cancer mortality rate that has occurred during the past 20 years. Currently available treatments of HCC are largely inadequate. However, with better understanding of the molecular pathogenesis of the cancer and significant advances in gene silencing technology, improved approaches are being devised to counter the malignancy. In particular, harnessing the RNA interference (RNAi) pathway to inhibit expression of genes that are implicated in transformation of hepatocytes is an exciting new approach to treating HCC. Therapeutic gene silencing technology is however at an early stage of development and there are several important hurdles that need to be overcome before this approach becomes a reality for treating HCC. In this review, we summarize HCC molecular pathogenesis as a background to discussing the interesting prospects of RNAi-based drugs for treating HCC.

CAUSES AND MOLECULAR PATHOGENESIS OF HCC

There are several etiological agents and risk factors that

have been implicated in causing HCC^[2]. Typically, HCC arises within a diseased liver and chronic liver injury *per se*, usually cirrhosis is thought to be causative of HCC. Thus, agents that result in chronic liver disease, although perhaps not directly carcinogenic, may be risk factors for HCC. Liver cancer is not evenly distributed throughout the world and is a consequence of unequal prevalence of major causative factors of the malignancy^[2]. HCC has a particularly high incidence in sub Saharan Africa, East and South East Asia where chronic hepatitis B virus (HBV) infection is common. Of the factors that have been identified to cause HCC, persistent HBV infection has the strongest association with the malignancy^[1]. The long term risk of HCC in HBV carriers has been reported to be in the range of 25%-40%. Infection with hepatitis C virus (HCV) is also directly causative of liver cancer^[4]. The long-term risk for HCC in HCV-infected individuals is estimated to be 1%-3% after 30 years, although in cases of established HCV-related cirrhosis, the annual rate of HCC development may be as high as 7%. Globally it is estimated that there are 387 million carriers of HBV^[1,5] and 170 million people persistently infected with HCV^[6]. These enormous numbers make HBV and HCV chronic infections the major HCC-predisposing factors. A primary focus of preventing HCC is thus aimed at eliminating these viruses.

Several other hepatocarcinogenic factors, which may cause transformation directly or indirectly, have been identified^[2]. These include excessive alcohol intake, aflatoxin ingestion, vinyl chloride exposure, obesity, diabetes mellitus, dietary iron overload, cigarette smoking and use of oral contraception. Regardless of the study population, males have a higher risk for the malignancy than females. The male to female ratio of HCC varies between 2:1 and 4:1. A reason for this gender bias is a higher risk for exposure to HCC-causing agents such as alcohol and tobacco amongst males. Additionally, androgens *per se* may contribute to hepatocarcinogenesis.

HBV

HBV is the prototype member of the hepadnaviridae family of hepatotropic viruses. It is a small, non-cytopathic, enveloped partly double stranded or relaxed circular DNA (rcDNA) virus with a genome size of 3.2 kb^[7-9]. Viral rcDNA is converted to covalently closed circular DNA (cccDNA) within an infected hepatocyte. cccDNA then serves as template for expression of viral genes, formation of pregenomic RNA and ultimately the production of progeny viruses. The viral genome is remarkably compact and encodes four overlapping open reading frames (ORFs): core (C), polymerase (P), envelope (surface, S) and X (HBx), which collectively encompass the entire genome. Viral cis elements that control transcription and aspects of replication are thus embedded within protein coding sequences. This remarkably economical use of the small genome limits HBV sequence plasticity and the virus is thus a good target for therapy that is based on nucleic acid hybridization.

The exact mechanism of HBV-mediated hepatocarcinogenesis is not completely understood. Integration of viral DNA into the host cellular genome with resultant

effects on chromosome stability and surrounding genes may play a role. Several lines of evidence also implicate HBx in the transformation of hepatocytes. This protein has wide ranging effects on cellular processes that are involved in regulating cell differentiation, apoptosis and proliferation. The mechanism of action includes indirect transcriptional activation of cellular *cis* elements, effects on cell signalling pathways as well as modulation of apoptosis^[10].

HCV

HCV is a member of the Flaviviridae family. The virion is enveloped and has an RNA genome comprising 9.6 kb of uncapped RNA with sense polarity^[11]. The internal ribosomal entry site (IRES), which is located within the 5' NTR of the HCV genome, initiates translation of a large precursor polyprotein. This precursor is processed by cellular and viral proteinases to form 10 viral proteins, namely core, E1, E2, p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B. E1 and E2 encode the envelope proteins and the viral core is derived from the C sequence. Non-structural proteins are responsible for assembling the viral proteinase machinery (NS2, NS3 and NS4A), forming the specialized membrane compartment for viral replication (NS4B) and RNA-dependent RNA polymerase (NS5B). The hydrophobic p7 protein, required for the late stages of viral assembly, is thought to function as a viroporin. The frameshift (F) protein, which is expressed during natural HCV infection, is yet to be fully characterized^[12-14]. The entire HCV lifecycle is cytoplasmic and involves the formation of minus RNA and dsRNA intermediates within the membranous web. HCV dsRNA activates the innate immune response, but cellular antiviral effects are inhibited by the E2, NS3 and NS5A proteins^[15-17]. Unlike with HBV, sequence heterogeneity and rapid evolution of quasispecies are characteristic of HCV infection. However, structural characteristics of the 5' NTR, which determine its function as an IRES impart sequence conservation in this region^[11].

Research suggests that HCV exerts its oncogenic effect through the actions of its viral proteins. The amino terminal of the NS3 protein^[18] and the core protein^[19] have been shown to influence various cellular functions including the enhancement^[20-22] or inhibition^[23-25] of apoptosis. The core protein may activate transcription of the proto-oncogene *c-myc* and also has an effect on apoptosis through effects on Fas, tumor necrosis factor (TNF-)^[26] and a mitogen activated protein kinase or extracellular signal regulated kinase (MAPK/ERK) signaling cascade. In addition, the NS4B protein functions as a transcriptional activator of intracellular signals that play roles in cell proliferation and inflammation. NS3 and NS5A proteins may also contribute to hepatocarcinogenesis by blocking the action of p53^[27,28].

Aflatoxin and ethanol

Aflatoxin exposure and excessive alcohol intake are important causes of liver cancer, which may have synergistic action when occurring together with chronic HBV and HCV infection^[29]. Aflatoxins are mycotoxins generated by certain *Aspergillus* species and are potent natural carcinogens^[30]. When converted to exo-8,9-epoxides in

the liver they are capable of damaging guanine nucleotides in hepatocytes DNA to form mutagenic and potentially carcinogenic DNA adducts. Importantly, aflatoxin-induced mutation hot spots in the liver include a transversion that occurs in the third position of codon 249 of the p53 tumor suppressor gene^[31,32]. Alcohol metabolism in the liver has also been implicated in mutagenesis and hepatocarcinogenesis^[33]. The mechanism is through generation of reactive oxygen species (ROS) and increasing hepatic oxidative stress. Acetaldehyde accumulation also contributes to the formation of protein and DNA adducts which result from ROS generation. Excessive alcohol intake is also a cause of chronic liver injury and cirrhosis, which is itself indirectly hepatocarcinogenic.

CURRENT TREATMENT OF HCC

Although there have been advances in the detection of HCC, improvement in the management of the disease has not been significant^[34]. Inadequacy of current therapy and presentation of patients with advanced disease have meant that the prognosis of individuals with HCC remains poor. Available treatment modalities include surgical intervention, percutaneous and arterial attempts at tumor ablation as well as drug-based treatment. If patients have well localized malignancies and good underlying liver function, surgical resection does have some success in eradicating tumors. However, this is clinically atypical, and patients with HCC usually present with cancers that have spread extensively within the liver and to distant sites. Treatment in these cases is palliative and the prognosis is extremely grave. Very early tumor detection and novel effective therapy are important objectives for improving management of patients with HCC. The potent targeted gene silencing that can be achieved by activation of RNAi has prompted investigations that apply this approach to direct treatment of HCC as well as through eliminating risk factors for the malignancy (e.g. HBV and HCV).

HARNESSING RNAI TO TREAT HUMAN DISEASE

RNAi is a powerful gene silencing mechanism that operates in most eukaryotic cells^[35]. The effector molecules comprise short duplex RNA sequences of 21-23 bp that direct inhibition of homologous genes (Figure 1). Naturally, the pathway is important for processing of regulatory micro RNAs (miRs)^[36,37]. These non-coding cellular sequences have roles in several pathways such as cell differentiation, metabolism, proliferation and malignant transformation. The miR processing pathway is initiated by transcription of miR-encoding cellular genes by RNA polymerase II (Pol II) to produce hairpin-containing primary miRs (pri miRs). These pri miRs may be derived from intronic sequences and may be polycistronic. Within the nucleus, pri miRs are processed to form precursor miR (pre-miR) hairpins of 60-80 nt in length. This step is catalyzed by the microprocessor complex, which contains Drosha and di George Critical

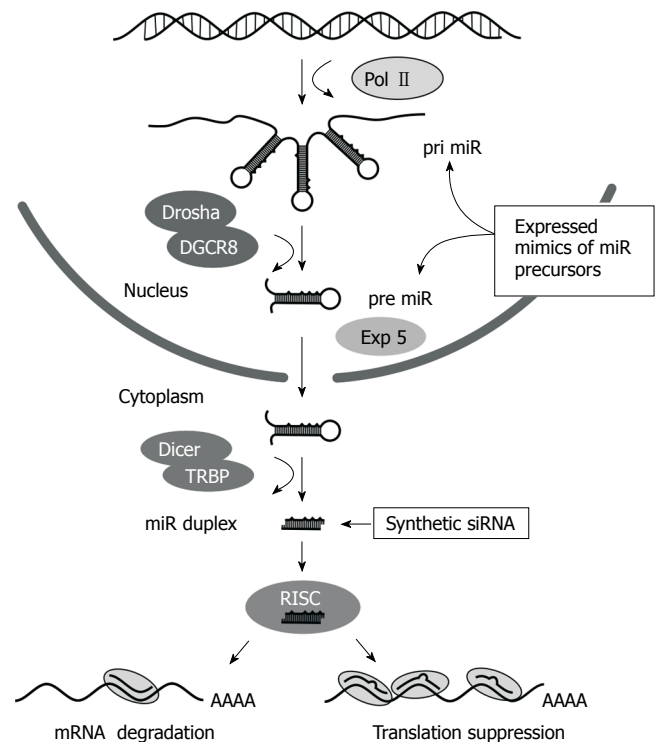


Figure 1 Schematic illustration of the RNAi pathway showing the essential steps, with nuclear or cytoplasmic location, involved in micro RNA processing. Exogenous activators of the pathway, which may be synthetic siRNA or expressed mimics of miR precursors, are shown.

Region 8 (DGCR8) proteins. Drosha functions as an RNase III enzyme and DGCR8 is its double stranded RNA binding protein partner. Pre-miRs are exported from the nucleus to the cytoplasm by the RanGTP-dependent Exportin 5 transporter. Pre-miRs are then processed by Dicer with associated TAR RNA-binding protein (TRBP) to form a staggered RNA duplex of 21-24 bp with 2 nt 3' overhangs. This duplex is handed on to the RNA induced silencing complex (RISC), which includes several components such as Argonaute 1 (AGO1), Argonaute 2 (AGO2) and Fragile X proteins. One strand of the RNA duplex, which is designated the passenger strand, is cleaved within RISC and is then released from the complex. The remaining intact single stranded guide RNA activates RISC to direct target-specific silencing. Mature cellular miRs are usually not entirely complementary to their targets and bind to the 3' untranslated regions of cognate mRNA to induce translational suppression. Hybridization between target and nucleotides 2-8 from the 5' end of the guide strand, termed the seed sequence, are all that is required to cause translational suppression^[38]. When base pairing between entire guide and target is perfectly matched, the AGO2 component of RISC exerts silencing through site-specific cleavage ('slicing') of the guide complement^[39,40].

Exogenous activators of RNAi may be used for therapeutic application by silencing specific pathology-causing sequences. These activators are typically expressed or synthetic sequences and they activate RNAi at different stages of the pathway (Figure 1). Exogenous small interfering RNAs (siRNAs) are usually synthetic mimics of the dsRNA duplexes that are formed after Dicer

processing, whereas expressed RNAi effectors are typically designed to simulate pri miR or pre miR sequences. The commonest form of expression cassette comprises a short hairpin RNA (shRNA)-encoding sequence that is inserted downstream of a Pol III promoter. When introduced into a cell, the shRNA acts as a mimic of pre miR and is processed by Dicer to form a siRNA. Synthetic or expression cassette-derived siRNAs are structurally symmetrical molecules but stability at the 5' ends of the siRNA duplex has an influence on the bias of guide strand selection^[41]. The strand which is less tightly paired (A/U rich) to its complement at its 5' end is preferentially incorporated into RISC. Other factors that influence the effectiveness of siRNAs against their cognate targets have been described and have been incorporated into predictive algorithms used for design of exogenous silencing sequences. The U6 and H1 Pol III promoters have commonly been used to generate expressed shRNAs and they are capable of efficient transcription of short defined sequences. Importantly, Pol III promoters are usually constitutively active, which means that regulation of intracellular concentration of shRNAs is difficult to achieve^[42]. Related to this, recent demonstration that U6 Pol III-expressed shRNAs may saturate the endogenous miR pathway to cause serious toxicity *in vivo* is an important concern^[43]. Since naturally occurring pri miRs are expressed from Pol II promoters^[44], shuttle cassettes incorporating features of pri miRs may improve the efficiency of Pol II-expressed RNAi sequences.

HCC AND MIRS

There are several studies that have implicated disruption of miR expression in carcinogenic mechanisms^[45,46]. miR concentrations may be elevated or depressed in HCC, which suggests that by interacting with their cognates, these sequences may act as oncogenes or suppressors of hepatocyte transformation. Recent studies using miR microarrays, showed high expression of miR-21^[47] and low levels of miR-122a^[48] in HCC. miR-21 contributes to hepatocyte transformation, growth and spread by inhibiting phosphatase and tensin homolog (PTEN) tumor suppressor. Modulation of cyclin G1, a cell cycle modulator, is the mechanism by which decreased miR-122a expression was reported to be hepatocarcinogenic. Another study analyzing the role of peroxisome proliferator-activated receptor alpha (PPARalpha) in hepatocarcinogenesis showed that this protein is an important regulator of hepatic miRNA expression^[49]. Of particular significance was PPARalpha inhibition of let-7C-mediated signaling and *c-myc* induction, which leads to hepatocyte proliferation and transformation. Other studies have implicated different alterations in miR expression that may be important for HCC^[50,51]. A recent study, which analyzed sequence variation of 59 miRs in HCC and adjacent non-malignant tissue, revealed four variations in three microRNAs^[52]. These miRs included miR-106b, miR-192 and let-7a-2. The significance of the variations for hepatocarcinogenesis was however not clear as the same sequence differences were found in non-malignant cells but not in eight liver cancer-derived cell lines. The

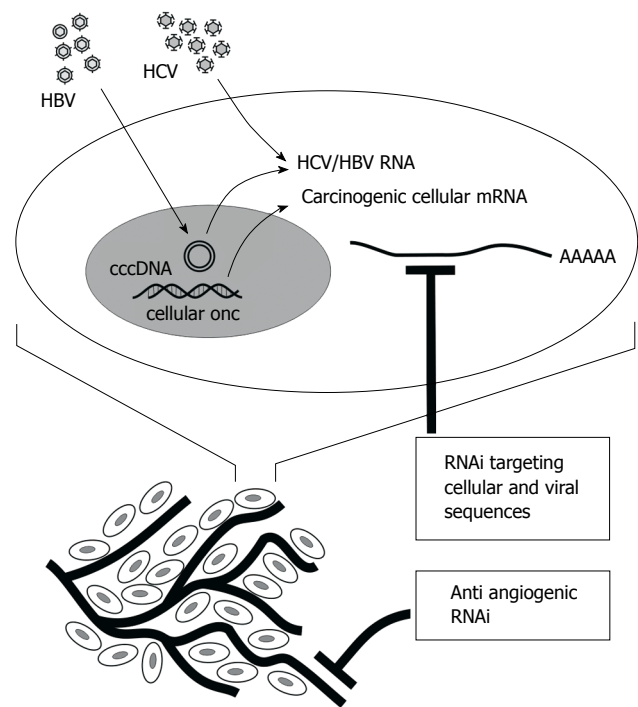


Figure 2 RNAi targets that may be silenced to counter HCC. In addition to HBV and HCV genes, cellular sequences that are involved in hepatocyte transformation may be silenced to inhibit growth of malignant liver cells. Growth of HCC is also dependent on angiogenesis and inhibition of this process is expected to limit tumor growth.

conclusion from this study was that miR mutation is a rare event in HCC and is unlikely to represent the main mechanism of hepatocarcinogenesis. Collectively the data show considerable heterogeneity in HCC-related altered miR expression. This complicates developing a generic approach to HCC treatment that is based on modulation of miR expression.

RNAI TARGETS TO COUNTER HEPATOCARCINOGENESIS

Suitable targets for development of RNAi-based treatment of HCC include cellular oncogenic sequences, angiogenic factors, as well as HBV and HCV genes (Figure 2). Studies to date, which have mainly demonstrated proof of principle, are summarized below. A difficulty of assessing usefulness of RNAi against HCC, HBV and HCV has been the lack of convenient small animal models. Advances have recently been made in addressing this obstacle. HCV replicons^[11], cells in culture that are permissive for HBV^[53] and HCV^[54] infection, the hydrodynamic injection procedure^[55], transgenic mice and xenografted mice^[56] have, and will be, particularly useful.

HBV

As with most investigations aimed at developing RNAi-based therapy, synthetic and expressed sequences have been used to activate RNAi and counter replication of HBV. Most studies to date have provided proof of principle in cell culture and small animal models of HBV replication. Recently however, FDA approval has been

granted for an investigational new drug license to test the use of expressed RNA sequences against HBV (http://www.nucleonicsinc.com/news/pdfs/FDAClearanceHepB_Trial_may2007.pdf).

One of the first studies aimed at assessing efficacy of RNAi-activating sequences against HBV tested a panel of six U6 promoter cassettes that encoded shRNAs^[57]. Profound inhibition of HBV surface antigen secretion from transfected cultured cells and *in vivo* in the murine hydrodynamic injection model was observed. Inhibitory effects were found to be due to a direct effect that is not dependent on an antigen-dependent immune response. Others have recently demonstrated varying degrees of inhibition of HBV replication by expressed shRNA sequences^[58-64] and one study showed antiviral synergy between lamivudine and shRNA sequences in a cell culture model^[61]. To address concerns about the emergence of RNAi escape mutants, Weinberg and colleagues^[65] used long hairpin RNA (lhrRNA) expression cassettes to target the HBx ORF of HBV. These sequences were designed to generate multiple siRNAs from the 62 bp duplex region of the hairpin. Although impressive knockdown was achieved, the efficacy was not equal across the span of the duplex. siRNAs generated from the stem base were produced more efficiently and effected better silencing than the loopside siRNA template. Limited processivity by Dicer, which initiates its RNase III activity at the hairpin stem base, is thought to be the cause of this effect. Using an approach that obviates this problem by cleavage of hairpin RNA *in vitro*, endoribonuclease-prepared siRNAs (esiRNAs) targeting all four HBV ORFs were recently used to inhibit HBV replication with high efficiency^[66].

Since there is no convenient small animal model of HBV infection, transgenic mice have been used in some studies as a stringent model to simulate virus replication that occurs in HBV chronic carriers. Expressed shRNAs have been tested in 4 studies on HBV transgenic mice^[43,62,63,67]. Hepatotropic recombinant adenovirus vectors expressing shRNAs from Pol III promoters caused sustained inhibition of viral replication over a 2-4 wk period after administration of a single dose of the vector^[62,63]. The importance of optimizing the dose of expressed anti HBV shRNA sequences was highlighted in a recent study that showed fatality in HBV transgenic mice that were treated with recombinant adeno-associated virus (AAV) type 8 vectors that over expressed shRNAs^[43]. Recombinant vectors expressing a 25-mer anti-HBV shRNA consistently caused death of treated mice, whereas low dose of a 19-mer shRNA vector efficiently suppressed markers of viral replication. These studies provide important evidence that HBV is susceptible to RNAi-based gene silencing in a model that simulates established ongoing replication that occurs in HBV chronic carriers.

Effective knockdown of HBV replication by synthetic siRNA has also been shown *in vitro* and *in vivo*. A siRNA duplex that targeted sequences immediately upstream of the surface ORF initiation codon was found to be particularly effective against HBV without a requirement for HBV DNA synthesis^[68]. This property is distinct from anti-HBV nucleoside or nucleoside analogues, which act on

the viral DNA polymerase to have their therapeutic effect. Efficacy of *surface* ORF-targeted siRNAs was reported in other studies^[69,70]. Improved knockdown by repeated siRNA transfection of cells in culture was also observed^[71] as well as effectiveness against a HBV target that includes the sequences encoding the lamivudine-resistance YMDD *polymerase* gene mutation^[72]. Recently, Morrissey and colleagues showed that chemically modified siRNAs caused potent and persistent anti-HBV activity *in vivo*^[73]. Sequences were administered intravenously within a stable nucleic acid lipid particle (SNALP) formulation. Efficiency of the complexes is likely to be a result of increased stability *in vivo* and also diminished non specific immunostimulatory effects. Apolipoprotein A-1-derived nanoparticles have also been used successfully *in vivo* to deliver synthetic anti HBV sequences^[74]. Importantly, these vectors are liver specific and are efficient at low doses. As well as being developed as an independent therapy, RNAi effectors may be used in combination with established licensed drugs to improve efficacy. Such synergy, which is likely to result from different mechanisms of drug action, has been demonstrated when using anti-HBV sequences in conjunction with lamivudine^[61].

HCV

Since it is an RNA virus that replicates in the hepatocyte cytoplasm, HCV is considered a prime candidate for RNAi-based treatment. However, a high degree of heterogeneity of viral sequences has been a particularly significant obstacle to developing antiviral RNAi effectors. For this reason, the 5' NTR has been the favored HCV target of several studies^[75-79]. Also, the lack of suitable models of HCV reproduction *in vivo* has hampered development of RNAi-based approaches to therapy. Extensive use of subgenomic replicon systems has been used successfully to study efficacy of antiviral therapeutic agents in cell culture and this approach has provided valuable insights^[80,81]. Currently available models of HCV infection *in vivo* are limited and include the chimpanzee^[82] and chimaeric immunodeficient mice that are grafted with human hepatocytes^[83].

In an early study that employed RNAi against HCV^[79], both synthetic and expressed RNAi effectors against the 5' NTR caused significant reduction of markers of HCV replication in a replicon model. Subsequent investigations targeting the 5' NTR showed suppression of viral gene expression by naked shRNAs^[78] and also synthetic siRNAs^[84]. Domain IV regions were found to be a particularly good target for RNAi-based HCV gene silencing^[79]. However, a report by Takigawa and colleagues^[77] showed that expressed shRNAs targeted to NS3-1 and NS5B were more effective than sequences against the 5' NTR. Other studies using both synthetic and expressed siRNAs also achieved significant inhibition of virus gene expression when targeting NS3 and NS5B sequences^[85,86]. Although good efficacy against HCV has been demonstrated, a major concern remains the ability of the virus to accumulate evading nucleotide sequence mutations. Not surprisingly, emergence of resistant HCV replicons has been shown in cultured cells after repeated treatments with siRNA targeted against the

NS5B coding region^[87]. Point mutations within the siRNA target sequence were observed, but resistant replicons were sensitive to a siRNA that targets another part of the genome. As with HBV^[65], vectors that express lhrNAs have been shown to be effective against HCV^[88,89]. Although these sequences have the theoretical advantage of generating multiple siRNAs, the approach may be limited by incomplete Dicer processing of the hairpin (see above and^[65]). Although approaches that produce polycistronic miR shuttles or esiRNAs may be preferable, a concern of using multiple siRNAs for therapy is the increased likelihood of silencing non-targeted genes as well as disruption of the endogenous miRNA pathway.

Other studies have aimed to circumvent the problem of viral escape by targeting host genes that are required for HCV replication^[90-92]. Synthetic siRNA and adenovirus-delivered effectors specific for La autoantigen (La), polypyrimidine tract-binding protein (PTB), cyclophilins and human VAMP-associated protein of 33 kDa (hVAP-33) substantially blocked HCV replication. Endogenous hepatic miR-122 has recently been shown to suppress haem oxygenase-1 (HO-1) and facilitate HCV replication^[93,94]. Upregulation of HO-1 or suppression of miR-122 thus represents an interesting strategy to counter HCV infection. Caspase 8 and the Fas cell death receptor, which mediate T-cell hepatocyte toxicity caused by viral infection, were efficiently silenced using RNAi^[95,96]. Although promising, it remains to be established whether silencing of these endogenous genes causes toxicity.

Cellular oncogenic sequences

As the molecular mechanism of hepatocarcinogenesis becomes better understood, so the number of potential targets that can be inhibited using RNAi improves. There are many oncogenes that have been described, which are implicated in the disruption of control of normal hepatocyte proliferation. Although this is encouraging, the targeting of specific cellular sequences is hampered by two main factors: (1) heterogeneity of gene expression in liver cancer cells from different sources, and (2) difficulty of achieving sufficient transfer of RNAi effectors to be of therapeutic benefit against the malignancy. The possibilities for therapeutic application are nevertheless intriguing.

A recent study aimed to analyze the effect of silencing the pituitary tumor transforming gene (PTTG) family on hepatocarcinogenesis^[97]. PTTG is a recently discovered group of oncogenes that plays a role in the genesis of several types of cancer through effects on cell division, apoptosis and DNA repair^[98]. PTTG1, but not PTTG2 and PTTG3, is frequently over expressed in patient liver cancer tissue as well as in established HCC lines^[97]. Infecting cells with a recombinant adenovirus expressing an anti PTTG1 RNAi effector depleted cells of PTTG1 and resulted in the activation of p53 with consequent increased p21 expression and apoptosis. Inhibition of tumor growth in a nude mouse xenograft model of HCC further supported the notion that PTTG1 is a good candidate for RNAi-mediated HCC therapy.

Other studies aimed at silencing the serine protease urokinase-type plasminogen activator (u-PA) demonstrated

similar proof of principle efficacy^[99,100]. Signalling through u-PA and its receptor (uPAR) have been implicated in cell invasion, survival, and metastasis of a variety of cancers^[101,102]. Silencing of u-PA using RNAi-based approaches has been used successfully in tumor models of prostate cancer^[103] and gliomas^[104]. To assess efficacy of RNAi-based u-PA silencing on HCC, Salvi and colleagues^[100] demonstrated that stable expression of a shRNA effectively knocked down u-PA. Moreover, silencing of u-PA resulted in attenuation of malignancy-associated cellular properties, such as migration, invasion and proliferation. In a follow up study, the effects of stable inhibition of u-PA on xenografted tumor cells were assessed^[99]. Cells that stably produce silencing sequences targeted to u-PA were injected subcutaneously into nude mice. Knockdown of both u-PA protein and mRNA concentrations was observed, which lasted for a period of 11 weeks. A delay in xenografted tumor growth was observed in cells expressing anti u-PA sequences, which indicates that u-PA silencing may be beneficial for HCC therapy.

A further study aimed at developing RNAi-based HCC therapy, assessed inhibition of function of human gankyrin gene product (p28GANK)^[105]. This novel oncogenic protein is ubiquitously over expressed in HCC and plays a role in cell cycle progression in normal hepatocytes and liver regeneration^[106-109]. After screening for susceptible target sites, a shRNA expression cassette was incorporated into an adenoviral vector. This was used to determine silencing of p28GANK and assess antitumor properties of the viral vectors^[105]. Effective silencing of approximately 80% was achieved. This depletion of p28GANK enhanced dephosphorylation of Rb1 decreased transcriptional activity of E2F-1 in cultured liver-derived cells and inhibited cell growth. Moreover, tumor cell growth was retarded in xenografted nude mice, which was thought to be a result of increased caspase-8- and caspase-9-mediated apoptosis caused by p28GANK inhibition.

RNAi-based approaches to the inhibition of vascularization of tumors has recently received attention^[110-115]. HCC growth is dependent on neovascularization and inhibition of factors that are required for angiogenesis should therefore be effective in countering the growth of this cancer. Most work has focused on the silencing of vascular endothelial growth factor (VEGF) to reduce the formation of new vessels that are required for tumor development. RNAi-based inhibition of VEGF in cases of the wet form of macular degeneration has reached an advanced stage of clinical trial (<http://www.agingeye.net/maculardegen/maculardegennewdevelopments.php>). Although this disease is not a malignancy, demonstration that VEGF can be inhibited successfully *in vivo* using RNAi indicates that this target may also be suitable for clinical application to HCC therapy. Alnylam and Inex Pharmaceuticals, leaders in the field of developing RNAi-based human therapy, have recently advanced a combination systemic drug for the treatment of liver cancer (<http://www.alnylam.com/therapeutic-programs/programs.asp>). The therapeutic, which is a liposomal formulation termed ALN-VSP-1, contains siRNAs that

target VEGF and kinesin spindle protein (KSP). siRNA-mediated inhibition of KSP production leads to cell cycle arrest and death in malignant hepatocytes.

CHALLENGES FACING USE OF RNAI-BASED THERAPY FOR TREATMENT OF HCC

Although the studies summarized above indicate that RNAi could be used for preventative or curative HCC treatment, there are several important hurdles that need to be overcome before clinical application. These include activation of the innate immune response, limitation of unintended interaction of RNAi effectors with cellular sequences, dosage regulation and optimizing delivery methods. These topics are briefly summarized below.

Innate immune response activation

Duplex RNA within cells is sensed as unwanted gene activity and may result in unintended harmful effects caused by activation of inflammatory cytokines and the interferon (IFN) response^[116-118]. Stimulation of cytoplasmic pattern recognition receptors, such as dsRNA dependent protein kinase (PKR), retinoic acid inducible gene-1 (RIG-I) and Toll-like receptors (TLRs), leads to a cascade of events, which culminates in activation of transcription factors such as NF- κ B, IRF3 and IRF7. This in turn causes increased expression of genes that include inflammatory cytokines and interferons. The response may be further amplified by autostimulatory positive feedback that involves JAK-STAT pathway activation. IFN pathway activation may also lead to inhibition of protein synthesis and degradation of cellular mRNA with consequent programmed cell death (apoptosis). The type of effector sequence that is used to activate RNAi also has a bearing on immunostimulation^[119]. Synthetic siRNAs that are longer than 30 bp^[120], possess 5' triphosphates^[121] and lack 2 nucleotide 3' overhangs^[122] stimulate the innate immune system. Also, 'danger' motifs (e.g. GU rich sequences, 5'GUCCUCAA3' and 5'UGUGU3') may activate endosomal TLR3, TLR7 and TLR8^[123]. RNAi effectors derived from expression cassettes that are transcribed from the nucleus do not pass through the endosomal compartment to activate these TLRs. However, unmethylated CpG islands within RNAi-activating DNA expression cassettes may be immunostimulatory. Recently, chemical modifications of siRNAs have been shown to attenuate immunostimulatory effects^[124], and this has been used successfully *in vivo* to counter HBV replication without release of interleukins and inflammatory cytokines^[73].

Non-specific interaction of silencing molecules with cellular sequences

Cross-hybridization of siRNAs with transcripts that have partial sequence identity^[125], particularly in the seed region of the intended target, may contribute to non-specific silencing effects. An interesting recent observation has been that 2' O-methyl ribosyl modification at position 2 of the siRNA guide strand reduces off target silencing at the seed site^[126]. This effect was independent of the

target and did not influence knockdown efficiency of perfectly matched sequences. Incorporation of HCC-specific transcriptional regulatory elements (e.g. the alpha fetoprotein promoter) may be helpful to improve specificity of expressed RNAi effector by limiting transcription to malignant hepatocytes. This approach has been used successfully to accomplish tumor-specific transgene expression^[127-129]. In the long term, to address the problem of off-target silencing it is likely that detailed microarray analysis of cellular transcripts will need to be undertaken as part of developing RNAi-based HCC therapy.

Optimizing delivery vectors

One of the most difficult challenges impeding the advancement of RNAi-based HCC therapy is efficient and safe delivery of effector sequences. Ideally, vectors should deliver silencing molecules selectively to most if not all the malignant hepatocytes. Synthetic siRNAs are smaller than DNA expression cassettes. They do not require delivery to the nucleus and activate RNAi within the cytoplasmic compartment. This makes regulated non-viral vector (NVV)-mediated delivery of synthetic siRNAs easier to achieve than it is for larger DNA expression cassettes. Viral vectors incorporate expression cassettes of necessity, and are generally more efficient vehicles *in vivo* than NVVs. Ease of scalable synthesis and chemical modification to influence biological properties are important properties of NVVs that, with improved delivery efficiency, are likely to contribute to their gaining favor for clinical application.

Recombinant adenoviruses and adeno-associated viruses (AAVs) are capable of transducing liver cells with high efficiency and have been used successfully to deliver sequences that silence HBV or HCV gene expression^[43,62,63,90]. Despite recent concerns^[130], recombinant AAVs are attractive vectors as they appear to be safe and capable of long-term gene expression. They are also relatively easy to propagate and capsid sequences from naturally occurring hepatotropic AAV serotypes have been used to confer liver-targeting properties on the recombinant viruses^[131]. Although efficient delivery vehicles, adenoviruses are strong stimulators of innate and adaptive immune responses. This may cause toxicity and limit repeated administration^[132]. An added problem is that malignant hepatocytes may be refractory to infection with recombinant adenovirus vectors^[128]. To modify tropism and reduce immune responses, recent studies have used surface modified^[133] or helper-dependent 'gutless' vectors^[134]. Surface modification of adenovirus vectors with synthetic polymers such as poly-N-(2-hydroxypropyl) methacrylamide (poly-HPMA) and polyethylene glycol (PEG) has been used to facilitate tissue targeting and diminish immunostimulatory protein-protein interactions. Helper dependent and chemically modified vectors may have an improved safety profile that could be better suited to clinical application. An interesting effect of adenoviruses on miR biogenesis, which may have an influence on their suitability of use as vectors for RNAi-based therapy, is mediated by the virus associated RNA (VA1)^[135]. This RNA sequence of approximately 160 nt

folds into a structure that mimics miR and has the effects of reducing nuclear export of pre miRs by saturating exportin 5 and acting as a competitive inhibitor of Dicer.

NVV nanoparticles have been used effectively to target HCC cells and also to deliver anti-HBV/HCV sequences. Modifications to confer HCC specificity include incorporation of HBV L protein^[136] as well as epidermal growth factor (EGF)^[137] into the complexes. Proof of principle has been demonstrated in xenografted models of HCC, but efficiency of these vectors may not yet be adequate for therapeutic gene transfer. SNALPs containing synthetic siRNAs have been used successfully to inhibit HBV replication in a murine model of virus replication^[73] and also to silence endogenous hepatic gene expression in primates^[138]. A recent study investigated the use of a lactosylated cationic liposome 5 (CL-LA5) vectors to deliver anti HCV siRNAs to cultured cells and *in vivo*^[139]. The complexes accumulated in the liver and specifically suppressed intrahepatic HCV gene expression in transgenic mice. SNALP technology, CL-LA5 vectors and the recently described siRNA Dynamic PolyConjugates^[140] are interesting new vectors that offer exciting possibilities for future clinical application to HCC treatment.

Regulating dose

Lethal toxicity *in vivo*, which was caused by saturation of the endogenous hepatocyte miRNA pathway has come as an important warning for the development of RNAi-based treatment^[43]. Grimm *et al* showed that expressed shRNA sequences disrupted essential endogenous miRNA-mediated cell functions by saturating the rate limiting activity of exportin-5. Synthetic siRNAs should bypass this step and this has recently been shown to be the case^[141]. Dosage and intracellular copy of shRNAs is difficult to achieve with the commonly used constitutively active Pol III promoters, such as U6 and H1. Use of tissue-specific and inducible Pol II promoters may go some way to refining transcription control and improving dosage of expressed RNAi sequences.

CONCLUSIONS AND PROSPECTS

The enormous therapeutic potential of RNAi-based specific gene silencing has prompted enthusiasm for advancement of novel therapies for difficult to treat diseases, such as HCC. Developments have been exciting, and clinical trials are now in progress for treating a variety of diseases^[142]. However, use of gene silencing technology to treat established HCC faces major difficulties. These include identification of optimal targets, efficient and safe delivery of RNAi sequences and limitation of unintended off target effects. Hepatocarcinogenesis is a multistep process and because of the considerable heterogeneity underlying the molecular mechanisms of hepatocyte transformation, identification of ideal RNAi targets to treat the malignancy is complicated. HCC also often presents as a disseminated cancer and safe delivery of RNAi effectors to all malignant cells will require improvement of currently available vectors. Good progress has been made with silencing of HBV and HCV

replication using RNAi. It is likely that treatment of these virus infections, as an HCC preventative measure, will be the first RNAi-based therapies to counter the malignancy. Currently, early diagnosis of HCC is critical for its effective treatment. Success of RNAi against HCC is also expected to be dependent on identifying the malignancy in its early stages before tumor bulk becomes excessive. In the near future, it seems that silencing technology may well be used as an adjunct to other liver cancer treatments. Thus, the utility of RNAi-based therapy is also likely to be reliant on improvement of existing treatment and diagnostic modalities. Despite these difficulties, intensive efforts from several quarters have given momentum to development of RNAi-based HCC therapy. It is difficult to anticipate technological advancements, but the field is likely to see considerable progress during the coming years.

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TOPIC HIGHLIGHT

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Is human hepatocellular carcinoma a hormone-responsive tumor?

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Abstract

Before the positive results recently obtained with multitarget tyrosine kinase inhibitor sorafenib, there was no standard systemic treatment for patients with advanced hepatocellular carcinoma (HCC). Sex hormones receptors are expressed in a significant proportion of HCC samples. Following preclinical and epidemiological studies supporting a relationship between sex hormones and HCC tumorigenesis, several randomized controlled trials (RCTs) tested the efficacy of the anti-estrogen tamoxifen as systemic treatment. Largest among these trials showed no survival advantage from the administration of tamoxifen, and the recent Cochrane systematic review produced a completely negative result. This questions the relevance of estrogen receptor-mediated pathways in HCC. However, a possible explanation for these disappointing results is the lack of proper patients selection according to sex hormones receptors expression, but unfortunately the interaction between this expression and efficacy of tamoxifen has not been studied adequately. It has been also proposed that negative results might be explained if tamoxifen acts in HCC *via* an estrogen receptor-independent pathway, that requires higher doses than those usually administered, but an Asian RCT conducted to assess dose-response effect was completely negative. Interesting, preliminary

results have been obtained when hormonal treatment (tamoxifen or megestrol) has been selected according to the presence of wild-type or variant estrogen receptors respectively, but no large RCTs are available to support this strategy. Negative results have been obtained also with anti-androgen therapy. In conclusion, there is no robust evidence to consider HCC a hormone-responsive tumor. Hormonal treatments should not be part of the current management of HCC.

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Key words: Hepatocellular carcinoma; Sex hormones; Hormonal treatment; Tamoxifen

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the sixth most common cancer worldwide in terms of incidence (626 000 new cases per year, representing 5.7% of new cancer cases). Due to its very poor prognosis, the number of deaths is almost the same (598 000), representing the third most common cause of death from cancer^[1]. The overall sex ratio (male:female) is around 2,4. HCC causes more deaths in men (416 882 deaths in 2002, ranking third cause of cancer death) than in women (181 439 deaths, ranking sixth)^[1].

Treatment options and prognosis of patients diagnosed with HCC largely depend not only on tumor characteristics, but also on the severity of the underlying chronic hepatic

disease, which affects most of the patients^[2]. Prognosis is relatively better for the subset of patients eligible for surgical treatments (tumor resection, liver transplantation) or other loco-regional treatments with potentially curative aim (e.g., percutaneous ethanol injection, radiofrequency ablation). A worse outcome is expected in those patients who can be treated only with palliative loco-regional treatments (e.g., transarterial chemo-embolization) or who are not suitable for any of the above options.

Recently, sorafenib, a multi-target tyrosine kinase inhibitor targeting both the tumor cell and the tumor vasculature, has shown significant efficacy in the treatment of advanced HCC^[3]. Before the publication of these encouraging results, there was no systemic treatment that could be considered standard for advanced HCC^[2,4]. Cytotoxic drugs does not play a significant role in these patients: HCC cells show intrinsic resistance to chemotherapy, and treatment with these drugs is often associated with unacceptable toxicity, due to the often compromised liver function.

Normal human liver is morphologically and functionally modulated by sex hormones. Epidemiological studies in humans suggest that long-term use of oral contraceptives and anabolic androgenic steroids can induce both benign (hemangioma, adenoma, and focal nodular hyperplasia) and malignant (HCC) hepatocellular tumors^[5]. Animal models of experimental liver carcinogenesis suggest a relationship between exposition to sex hormones and development of HCC, with some evidence that these hormones may play a relevant role as inducer and promoter in the process of liver carcinogenesis^[6]. In typical animal models of hepatic tumor initiation and promotion following repeated estrogen stimulation, the estrogen induction of microsomally activated catechols by aryl hydrocarbon hydroxylase and estrogen 2-/4-hydroxylase causes excess free radicals and unrepaired DNA adducts and strand breaks, that produce a mutagenic and irreversible DNA damage. Several months after this tumor-initiating DNA damage, steroid receptors - estrogen receptor (ER), progesterone receptor (PgR), androgen receptor (AR) - increase well above normal levels, suggesting the relevance of sex hormones mediated pathways in cell growth and proliferation.

In the last decades, moving from this epidemiological and preclinical evidence, several clinical trials have tested the efficacy of hormonal treatment in patients with HCC. In this review, we summarize the evidence about the use of hormonal treatment in HCC, trying to understand if, in the era of target-oriented therapies, this disease can still be considered a potentially hormone-responsive tumor.

HORMONAL TREATMENT SHOULD BE STUDIED AS A TARGET-ORIENTED APPROACH

In addition to oncogenes, tumor-suppressor genes, and other genetic factors, a number of growth factors involved in cell signaling pathways have been shown to play a role in liver carcinogenesis^[7,8]. Angiogenic stimuli are also required for the growth of HCC, which is usually a hypervascular tumor.

Sex steroids are known to be able to stimulate cell growth directly in several cancer types. When the cellular mechanisms underlying autonomous tissue growth are linked to the growth-promoting effects of sex steroids, the clinical result is a *hormone-responsive* (also called *hormone-sensitive*) tumor. Hormonal treatment plays an established role in several solid tumors, like breast cancer and endometrial cancer in women and prostate cancer in men. As a matter of fact, hormonal treatment represents the first form of target-oriented cancer therapy, acting by disruption of growth factor (in this case, sex hormones) - receptor interactions. For example, binding of estrogen to ER induces activation of the receptor. In fact, ER, that resides in the cytosol, upon occupation by estradiol, dissociates from heat shock proteins and undergoes conformational changes, dimerization and phosphorylation^[9]. The activated ER is transported to the nucleus, where it binds to estrogen response elements that are located upstream of estrogen-regulated genes, including those encoding molecules involved in cell proliferation.

The potential role of a target-oriented approach in the treatment of a human tumor can be adequately evaluated only if three relevant points are properly considered: (1) the molecular epidemiology of the target in the proposed study population; (2) the role of the target in patho-physiology of the tumor; (3) the effectiveness of the available target-oriented drug on target inhibition. If we consider hormonal treatment of HCC as a target-oriented treatment, the three relevant points to be taken into account are: (1) presence of sex hormones receptors expression in HCC cells; (2) relevance of stimulation by sex hormones in human HCC proliferation; (3) effectiveness of available drugs on inhibiting hormonal receptors activation.

As for the last point, there is no doubt that very effective drugs are available. In the last decades, the anti-estrogen tamoxifen has been a mile-stone treatment for patients with hormone-sensitive breast cancer. Tamoxifen inhibits the growth of tumor cells by competitive antagonism of estrogen at its receptor site, and levels of estrogen receptor expression are the best predictor of benefit from tamoxifen^[10]. The real problem is that tamoxifen has been tested in patients with HCC, based on suggestive preclinical and epidemiological evidence, without an adequate evaluation of the first two points, that are even more important than the third one to enable the success of a target-oriented strategy: the target expression in HCC cells and the relevance of the sex hormones mediated pathways in HCC cell growth and proliferation mechanisms.

Expression of sex hormones receptors (ER, PgR and AR) - can be detected in a variable proportion of HCC^[11-19]. Table 1 shows the proportion of ER+, PgR+ and AR+ HCC in the studies analysing the expression of these receptors by different techniques, mostly enzyme immunoassay or immunohistochemistry. In the recent study by Vizoso *et al*, sex hormones receptors expression was determined in 31 HCC patients by immunohistochemistry using tissue micro-arrays^[19]. Their results demonstrate a wide variability in the immunohistochemical values for steroid receptors among HCCs: 67.7% of tumors stained positively for AR, 51.6%

for ER and 83.8% for PgR, but, among the positive cases, immunostaining score values for each protein were largely variable.

As for ERs, normal liver expresses almost exclusively wild-type ERs derived from the full-length transcript of the gene. Actually, there are two different ERs, ER- α and ER- β , that are produced by distinct genes. During progression of liver disease to HCC, variant forms of ERs have been demonstrated^[20]. Peritumoral cirrhotic tissue of patients with hepatocellular carcinoma, especially males, expresses a variant form of ER with an exon 5 deletion. This variant lacks the hormone-binding domain of the receptor but, being intact in the DNA-binding domain, maintains constitutive transcriptional activity. In HCC, variant ER largely predominates and sometimes becomes the only form expressed^[20]. The occurrence of variant ER alone is limited almost exclusively to males, and this suggests that it could be one of the molecular events that eventually lead to the preferential development of hepatocellular carcinoma in males. In addition, variant ER is more frequent in patients infected with the hepatitis B virus. The growth rate of HCC in patients with variant ER is also significantly higher than that in patients with tumors expressing wild type ER. These tumors with variant ER, that are a significant percentage of HCC, are characterized by a worse prognosis, with significantly shorter survival^[21].

EVIDENCE-BASED SUMMARY OF TAMOXIFEN EFFICACY IN HCC: RANDOMIZED CLINICAL TRIALS AND META-ANALYSES

Although only a limited percentage of HCC are ER+ and there is no robust evidence about the relevance of sex hormone-dependent pathways in HCC proliferation, in the last decades there has been great interest in the potential usefulness of tamoxifen for patients with HCC. Tamoxifen is characterized by a favourable tolerability profile, that, together with the easy oral administration, makes this drug an interesting candidate for treatment of solid tumors potentially responding to hormonal manipulation.

A number of randomized controlled trials have tested the efficacy of tamoxifen, with conflicting results (Table 2). Many of these studies were characterized by several methodological drawbacks, and by a really small sample size. Median survival in the control group was very variable, emphasizing the extreme variability in prognosis of patients with HCC.

With the aim of clarifying the benefit associated with tamoxifen and with other treatment strategies producing conflicting results in HCC, several systematic reviews have been conducted and published. Systematic reviews of health-care interventions are an attempt to collate information from all relevant studies and, if deemed appropriate, combine their results using meta-analysis. There have been four systematic reviews with meta-analysis of randomized clinical trials of tamoxifen in HCC^[34-37].

The two earlier reviews^[34,35] were conducted and published about ten years ago, when only small-randomized

Table 1 Expression of estrogen receptors, progesterone receptors and androgen receptors in HCC

Study	Number of cases	Ethnicity	ER (%)	PgR (%)	AR (%)
Nagasue ^[11]	30	Japanese	40	NA	NA
Ohnishi ^[12]	8	Japanese	14	NA	50
Hamazaki ^[13]	22	Japanese	23	NA	19
Nagasue ^[14]	19	Japanese women	37	NA	37
Boix ^[15]	26	Western	15	0	54
Ng ^[16]	71	Chinese	24	14	NA
Jonas ^[17]	33	Western	39	18	NA
Liu ^[18]	66	Chinese	27	30	NA
Vizoso ^[19]	31	Western	52	84	68

ER: Estrogen receptors; PgR: Progesterone receptors; AR: Androgen receptors; NA: Not available.

trials were available. Both reviews showed a marginal increase in overall survival with the use of tamoxifen in advanced HCC, suggesting that the preclinical rationale supporting the use of hormonal therapy in HCC patients could translate in to some clinical benefit.

The systematic review by Simonetti *et al* identified and considered seven trials: two trials^[22,24] evaluated the addition of tamoxifen to chemotherapy, and the other five trials^[23,25-27,29] were designed to compare tamoxifen versus no treatment or placebo. In these latter studies, pooled odds ratio of surviving at 1-year for patients receiving tamoxifen was 2.0, with 95% confidence intervals (CI) 1.1-3.6. This means a statistically significant result favouring tamoxifen. However, considering the limited quality of the evidence, the authors of the meta-analysis suggested a note of caution in considering these results definitive, calling for a large randomized controlled trial to definitely address the issue of the efficacy of tamoxifen in HCC.

Similarly, also the authors of the other review noted that further large, well-designed trials were needed to definitely answer this question, because controversy persisted about tamoxifen efficacy^[33]. In fact, their meta-analysis, considering seven trials, showed a borderline survival benefit, but, in sensitivity analysis, the survival benefit of tamoxifen was no longer significant.

Two years before the publication of the first systematic review, in 1995, the Cancer of the Liver Italian Program (CLIP) Investigators started the CLIP-1 large randomized trial, with the aim of verifying whether earlier interesting but conflicting data on tamoxifen effect were confirmed in a larger study^[31]. A pragmatic approach was chosen for the conduction of the trial. Eligibility criteria were broad, and all HCC patients with a life expectancy longer than 3 mo were eligible. Overall survival was the only endpoint of the intent-to-treat analysis, no placebo was used in the control arm and follow-up was conducted according to the usual clinical practice of participating centers. Patients assigned to the experimental arm received oral tamoxifen, 40 mg daily, until death or inability to assume the drug. Overall, 496 patients were randomized. Patients were predominantly males, with underlying viral cirrhosis. About half of them had a well compensated liver function. The results of the trial, published in 1998, showed no

Table 2 Randomized clinical trials testing the efficacy of tamoxifen in HCC

Reference	Patients	Study characteristics				Enclosed in meta-analysis			
		Tamoxifen arm		Comparator		Simonetti	Mathurin	Llovet	Cochrane
		Treatment	Overall survival	Treatment	Overall survival				
Melia, 1987 ^[22]	59	Adriamycin 60 mg/m ² + tam 20 mg/d	Median: 6 wk	Adriamycin 60 mg/m ²	Median: 8 wk	X	X		X
Farinati, 1990 ^[23]	38	Tam 30 mg/d	Median: 36 wk	No treatment	Median: 8 wk	X			
Uchino, 1993 ^[24]	26	Cisplatin, adriamycin, 5-fluorouracil + Tam 25 mg/m ² per d + MPA 400 mg/m ² per d	1-yr: 44.5%	Cisplatin, adriamycin, 5-fluorouracil	1-yr: 33.0%	X	X		
Elba, 1994 ^[25]	22	Tam 60 mg/d	Median: 74 wk	Placebo	Median: 52 wk	X	X	X	X
Martinez, 1994 ^[26]	36	Tam 20 mg/d	Median: 261 d	Symptomatic treatment	Median: 172 d	X	X	X	X
Cerezo, 1994 ^[26]									
Castells, 1995 ^[27]	120	Tam 20 mg/d	1-yr: 51%	Placebo	1-yr: 43%	X	X	X	X
Coll, 1995 ^[28]	61	Tam 20 mg/d	1-yr: 24%	Placebo	1-yr: 25%		X		X
Manesis, 1995 ^[29]	85	Tam 30 mg/d + triptorelin	Median: 282 d	Placebo	Median: 127 d	X	X	X	
				Flutamide 750 mg/d + triptorelin	Median: 112 d				
Riestra, 1998 ^[30]	77	Tam 40 mg/d	1-yr: 30%	Placebo	1-yr: 37.8%			X	X
CLIP group, 1998 ^[31]	496	Tam 40 mg/d	Median: 15 mo	No treatment	Median: 16 mo			X	X
Liu, 2000 ^[18]	119	Tam 30 mg/d	Median: 44 d	Placebo	Median: 41 d			X	X
Chow, 2002 ^[32]	329	Tam 60 mg/d	3 mo: 41%	Placebo	3-mo: 44%				X
		Tam 120 mg/d	3 mo: 35%						
Barbare, 2005 ^[33]	420	Tam 20 mg/d	Median: 4.8 mo	Symptomatic treatment	Median: 4.0 mo				

Tam: Tamoxifen; MPA: Medroxyprogesterone acetate.

overall survival advantage deriving from the administration of tamoxifen^[31]. Median survival was 15 and 16 mo in the tamoxifen and the control arm, respectively. One-year survival probability was similar in the two arms, 55% and 56%, respectively. After adjustment for known prognostic factors, the relative hazard of death for patients receiving tamoxifen was equal to 1.07 (95% CI, 0.83-1.39). Considering that the sample size of the CLIP-1 trial was much higher than that of all the previous studies, it is not surprising that the results of this trial changed the results of the meta-analysis. The addition of the CLIP-1 data to the four previous trials considered in the review by Simonetti *et al* comparing tamoxifen alone versus no active treatment produced a pooled odds ratio of being alive at 1 year for patients receiving tamoxifen of 1.19 (95% CI, 0.88-1.61), and there was no more statistically significant advantage for tamoxifen.

After the publication of the CLIP-1 trial, two systematic reviews with meta-analysis have been published^[36,37]. Both did not show any survival benefit for patients assigned to tamoxifen.

In the systematic review conducted by the Barcelona-Clinic Liver Cancer Group, and published in 2003, seven RCT were considered eligible for meta-analysis of tamoxifen effect^[36]. Tamoxifen showed no survival benefit [odds ratio (OR), 0.64; 95% CI, 0.36-1.13, $P = 0.13$], and the authors noted that only the low-quality trials showed any benefits.

Similar results are described in the Cochrane meta-analysis^[37] that considered nine randomized trials (one testing two doses of tamoxifen) for a total of 1709 patients. Tamoxifen *versus* placebo/no intervention had no significant effect on overall survival [hazard ratio

(HR), 1.05; 95% CI, 0.94-1.16; $P = 0.4$], without statistical heterogeneity between the trials. Trials were classified according to the adequacy or inadequacy of three methodological components: generation of the allocation sequence, allocation concealment and blinding. Subgroup analysis showed that a trend in reduction of mortality with tamoxifen was limited to trials with one or less adequate/three methodological components (HR 0.82; 95% CI 0.60-1.12; $P = 0.2$), whilst tamoxifen showed no significant effect in trials with two adequate/three methodological components (HR, 1.00; 95% CI, 0.84 to 1.18; $P = 0.98$) and tended to increase mortality in trials with three adequate/three methodological components (HR, 1.15; 95% CI, 0.99-1.34; $P = 0.06$).

ARE THERE ANY SUBGROUPS OF PATIENTS WHO RECEIVE BENEFIT FROM TAMOXIFEN?

Almost ten years ago, Mathurin *et al* discussed the conflicting results obtained with tamoxifen in their systematic review, stating that one of the main objectives in the future should have been to identify, using clinical and biological factors, the subgroups of patients responding to tamoxifen^[35].

Clinical factors

As for the identification of clinical subgroups, updated results of the CLIP-1 study, published in 2002, confirmed the original negative result obtained in the overall study population, both in the subgroup of advanced patients and in those eligible for potentially curative loco-

regional treatments^[38]. In a more recent RCT, conducted in France, 420 patients with HCC and a prevalence of alcohol-related liver cirrhosis were randomized to receive tamoxifen or supportive care alone^[33]. Despite the negative result in the overall population, following a post-hoc unplanned subgroup analysis, French authors suggested that tamoxifen might be effective in a population of Okuda stage I or II, i.e. those patients without major hepatic insufficiency, and that new trials on tamoxifen are still warranted, at least in this subset of patients. It should be noted that subgroup analyses carry a relevant risk of false positive results, and their results should be always considered with great caution. However, we tried to validate the hypothesis generated by the French trial using updated data from the CLIP-1 randomised trial, but tamoxifen still resulted not effective both in patients with Okuda stage I - II and in patients with Okuda stage III disease or Okuda unknown^[39]. We also tested the same hypothesis in subgroups defined according to the CLIP score. CLIP score is actually the most widely accepted and validated prognostic score for HCC^[40-42], and it takes into account liver function measured by Child-Pugh category, portal vein thrombosis, and level of alpha-fetoprotein and tumor size. In the patients of the CLIP-1 study, results were negative again both in patients with good CLIP score (0-1) and in those with worse or unknown CLIP score^[39].

Biological factors

Hormonal treatment can be considered the first form of target-oriented cancer treatment. Greater emphasis should be probably given, when planning a clinical trial and interpreting its results, to the great impact that the molecular heterogeneity of tumors, affecting sensitivity to the experimental treatment, may have on the results of the trial^[43]. This concept has been seldom taken into account in the planning and the analysis of clinical trials with cytotoxic agents, but in our opinion it should be necessarily applied in clinical trials with molecular targeted agents and, similarly, with hormonal treatment. We can imagine the whole population of potentially eligible patients as divided in to two distinct groups: one characterized by sensitivity to hormonal treatment, that will potentially produce in these patients an outcome better than the control, and the other, on the contrary, characterized by insensitivity to hormonal manipulation, that will translate in the absence of difference in efficacy between hormonal treatment and control. The higher the proportion of the latter patients in the study sample, the lower the power of the clinical trial to show a potentially positive result.

In particular, even in the case of solid tumors that are definitely considered hormone-sensitive, or hormone-dependent, not all the patients will derive benefit from hormonal treatment. In breast cancer, the disparity in clinical response to tamoxifen between women with hormone receptor-positive disease and those with receptor-negative tumors clearly established the utility of hormone receptor status in identifying those likely and, equally important, those unlikely to benefit from endocrine therapy^[10]. In fact, it is now well established that the efficacy of hormonal treatment is relevant, but it is limited to patients with tumors expressing hormonal receptors.

This principle became the basis on which current clinical practice guidelines were established for the application of this treatment in breast cancer.

If we try to transfer these simple considerations to the hormonal treatment of HCC, it seems reasonable that a possible explanation of the negative results obtained with tamoxifen could stay in the lack of proper selection of the patients. In our opinion, it is really disappointing that none of the RCTs testing the efficacy of tamoxifen in HCC did select eligible patients according to hormonal receptors expression. The expression of these receptors is not so frequent in HCC, and the levels of expression in positive cases are largely variable. This might have diluted the positive effect of tamoxifen, potentially limited to a small subgroup of patients.

The only attempt of correlating target expression and efficacy of hormonal treatment in HCC patients comes from a secondary analysis of a Chinese randomized trial comparing tamoxifen versus no treatment for patients with advanced and otherwise untreatable HCC^[18]. Immunohistochemical tests for ER and PgR were performed on the tumor tissues obtained from a subgroup of patients enrolled in the study. Disappointingly, in that series, the tumor expression of sex hormones receptors did not seem to affect the efficacy of tamoxifen^[18]. However, it should be noted that, in that trial, (1) patients were not prospectively selected or stratified according to qualitative or quantitative hormonal receptors expression, (2) immunohistochemical determinations were performed only on a subgroup of 66 patients with adequate tissue specimen, out of 119 enrolled patients, and (3) the prognosis of the patients enrolled in that study was really dismal, with a median survival of 44 d *versus* 41 d, in the tamoxifen and control group, respectively. Adequately powered prospective phase III trials assessing the efficacy of tamoxifen in patients selected or stratified (prospectively or retrospectively) for ER expression have never been conducted.

Another intriguing hypothesis about the possibility that tamoxifen could be effective only in a selected subgroup of patients with HCC is related to the presence of variant estrogen receptors (vER)^[20]. Tamoxifen could not be effective in tumors with vER, because of its inability to bind the receptor, and this could contribute to justify tamoxifen lack of efficacy, considering that a relevant proportion of HCC patients have predominant vERs^[44]. In a small experimental experience^[45], anti-hormonal therapy of HCC was tailored according to the presence of wild-type or exon 5-deleted vER transcripts, limiting the administration of tamoxifen (at a daily dose of 80 mg) to patients with wild-type ER, and treating patients with vER with megestrol acetate, at the daily dose of 160 mg. Interestingly, tumor volume in all patients with wild-type ERs was halved after 9 mo of tamoxifen treatment, and the investigators concluded that choosing anti-hormonal treatment according to the presence of wild-type or variant ERs in the tumor definitely improves the response rate to tamoxifen.

Of course, in our opinion, these intriguing results are not sufficient to definitely claim the efficacy of tamoxifen in a selected subgroup of HCC patients. These preliminary

results still lack confirmation in adequately powered and designed randomized controlled trials, prospectively selecting patients with wild-type ER and randomizing them to receive tamoxifen or no treatment.

SUPPOSED MECHANISM OF ACTION OF TAMOXIFEN IN HCC: ER-DEPENDENT VS ER-INDEPENDENT PATHWAYS

Some years ago, it has been proposed that the positive results obtained in some of the early small trials with tamoxifen in HCC and the negative results of the majority of the other trials might be explained if activity of tamoxifen in HCC could be related to an ER-independent pathway^[46]. Several mechanisms have been proposed by which tamoxifen could act on HCC cells independently from the expression of ER: the interaction of tamoxifen and 4-hydroxy-tamoxifen with membrane phospholipids, with decrease in cell membrane fluidity and inhibition of adenylate cyclase, the inhibition of Protein Kinase C activity, the inhibition of calmodulin-dependent cAMP phosphodiesterase and the increase in Transforming Growth Factor beta1 levels, that can be obtained also in ER- cells^[46].

Interestingly, these mechanisms, potentially interfering with cellular pathways relevant to HCC proliferation, require much higher doses of tamoxifen than those used in most of the clinical trials conducted so far. In fact, tamoxifen is known to have therapeutic actions independent of ER status at higher doses (4-8 times higher than the dose established for ER-positive breast carcinoma)^[46]. Thus, high-dose tamoxifen would potentially have therapeutic actions on both ER-positive and ER-negative HCC. Although correlation between sex hormone receptor expression and efficacy of tamoxifen in HCC has never been adequately studied, Tan *et al.*, in 2001, called for a “paradigm shift” to dissociate the action of tamoxifen from the expression of ERs^[46]. They suggested that future trials with tamoxifen in HCC should have used higher doses of tamoxifen, at least four to eight-fold that of the dose intended to be efficacious in an ER-dependent mechanism. Moving from this intriguing hypothesis, a double-blind RCT was conducted in the Asia-Pacific region with 329 HCC patients, comparing tamoxifen versus placebo^[32]. Tamoxifen was given at two distinct doses (120 mg daily and 60 mg daily), in order to assess possible dose-response effect. Quite disappointingly, rather than indicating a dose-response effect in favor of tamoxifen, the analysis showed a significant detrimental effect for the higher dose of tamoxifen. Three-month survival rates were 44%, 41%, and 35%, respectively for the placebo, tamoxifen at 60 mg, and tamoxifen at 120 mg groups, with a statistically significant trend difference in survival across the 3 arms. There was a significantly higher risk of death in the tamoxifen 120 mg group compared with the placebo group (HR, 1.39; 95% CI, 1.07-1.81). The detrimental effect of tamoxifen seemed not to be related to a higher toxicity of the higher dose. The trial, indeed, was unable to identify significant toxicity due to tamoxifen, and the rate of reported treatment toxicity (3%) was extremely low,

without significant differences among the arms; however, the Authors cautiously postulated that the general rapid decline of patients with inoperable HCC could make it difficult to identify treatment toxicities.

Although the mechanism by which higher doses of tamoxifen seems to have a negative impact on the prognosis of HCC patients is not completely clear, the unexpected findings of the Asian trial are confirmed by the results of the Cochrane meta-analysis^[37]. In fact, with increasing dose of tamoxifen, there was an overall survival trend favouring the arm without tamoxifen. Namely, the HR for overall survival was lowest for trials of tamoxifen given at 20 mg daily (HR 0.88; 95% CI, 0.69-1.44; $P = 0.71$), higher in trials of tamoxifen given at 40 mg daily (HR 1.00; 95% CI 0.85-1.19; $P = 1.0$), even higher in trials of tamoxifen given at 60 mg daily (HR, 1.03; 95% CI, 0.81-1.31; $P = 0.8$), and highest in the single trial of tamoxifen given at 120 mg daily (HR, 1.29; 95% CI, 1.04-1.6; $P = 0.02$).

According to these results, we believe that, unfortunately, neither further trials are warranted with tamoxifen in HCC, nor any use in clinical practice should be considered because of its clear lack of efficacy.

EVIDENCE WITH OTHER HORMONAL TREATMENTS: MEGESTROL ACETATE AND ANTI-ANDROGENS

Megestrol acetate exerts its action on ER-pathways at post-receptorial level. Efficacy of megestrol acetate has been tested in HCC with vER, according to the hypothesis that in these tumors a progestin drug might work better than tamoxifen^[45,47]. A small prospective, randomized study assigned patients with advanced HCC characterized by variant liver ER to receive megestrol or placebo^[47]. Out of 133 patients diagnosed with HCC and screened for eligibility, 45 patients (33.3%) had variant ER transcripts demonstrated in the tumor and were enrolled in the study. Twenty-four patients were randomized to no treatment and 21 to megestrol at the daily dose of 160 mg. Median survival in untreated patients was 7 mo (95% CI, 3.01 mo -10.99 mo) versus 18 mo (95% CI, 13.47 mo-22.53 mo) in patients treated with megestrol ($P = 0.009$). According to the comment of the investigators, megestrol was associated with a remarkable increase in overall survival in patients with HCC characterized by variant ERs, who usually show a rapidly progressive disease, making a trial with this drug more than warranted. We believe that no firm conclusions on the effectiveness of megestrol acetate in that selected subgroup of HCC patients should be drawn, in the absence of adequately powered randomized trials. Such trials should select patients according to the presence of variant ER, randomizing patients to receive megestrol or no treatment.

Similarly to estrogens, there is some preclinical evidence supporting a positive influence of androgens on HCC growth, with a potential role of treatment with anti-androgens for patients with HCC. In tumor cells, androgen receptors seem to be present more frequently and in greater concentrations than estrogen receptors^[48]. Furthermore, experimental studies have suggested a

promoter effect of androgens on tumor growth^[49], which may be suppressed via anti-androgen treatment^[50] or castration^[51].

A randomized trial conducted in unresectable HCC by the European Organization for Research and Treatment of Cancer tested the efficacy of anti-androgen therapy^[52]. The trial was conducted according to a factorial two-by-two design: patients were randomized to receive pure anti-androgen (nilutamide 300 mg daily for 1 mo, then 150 mg daily), luteinizing hormone-releasing hormone (LHRH) agonist (goserelin acetate at 3.6 mg or triptorelin at 3.75 mg administered monthly by subcutaneous injection), both treatments or control. Unfortunately, neither pure anti-androgen nor LHRH agonist showed significant efficacy in terms of survival. Another randomized phase III trial, designed with the aim of assessing the effect of anti-androgens in patients with advanced HCC, was conducted by the French collaborative group GRETCH^[53]. Male patients with advanced HCC were randomized into 2 arms. Patients assigned to the experimental arm received leuprolerin (3.75 mg/mo subcutaneously), flutamide (750 mg orally daily), and tamoxifen (30 mg orally daily). Patients assigned to control arm received tamoxifen alone, considered as a standard treatment at the time of study planning. Between February 1994 and January 1998, 376 male patients were included. Median survival time was 135.5 d and 176 d in combination and tamoxifen groups, respectively ($P = 0.21$). Adjusted relative risk of death in the treated group was estimated 1.08 (95% CI, 0.87-1.33). In conclusion, no benefit in survival was found with anti-androgenic treatment in male patients with advanced HCC.

MOVING TOWARD OTHER SYSTEMIC TREATMENTS

In the last decades, although supported by weak and conflicting results, use of tamoxifen in patients with advanced HCC has been probably encouraged by the absence of other active systemic options.

In recent years, with the development of new drugs targeting growth factor receptor pathways or other cellular pathways potentially relevant to tumor cell proliferation, research efforts have been focused on targeted therapies. Recently, sorafenib, that is a small molecule tyrosine kinase inhibitor acting against Raf kinase, VEGF, PDGFR- β , c-KIT and Flt has shown efficacy compared to placebo in a randomized controlled study conducted in 602 patients with advanced HCC, in the setting of Child-Pugh A cirrhosis^[3]. A planned interim analysis concluded that the trial met its primary end point, demonstrating a statistically significant and clinically relevant better survival in the sorafenib arm, without striking difference in the incidence of serious adverse events. Most recently, a planned interim analysis found similar results favouring sorafenib in an Asia-Pacific regional Phase III trial of patients with advanced HCC, enrolling 226 patients from sites in China, Korea and Taiwan. Based on these results, sorafenib has been the first drug approved for treatment of HCC, by both US Food and Drug Administration and European Medicines Agency.

On the contrary, disappointing results of clinical trials that have tested the efficacy of hormonal treatment in HCC raise serious doubts about the real relevance of sex hormones mediated pathways in the clinical course of HCC. Hormonal treatments should not be part of the current standard management of patients affected by hepatocellular carcinoma.

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TOPIC HIGHLIGHT

Harry HX Xia, PhD, MD, Series Editor

Reactivation of the insulin-like growth factor- II signaling pathway in human hepatocellular carcinoma

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Abstract

Constitutive activation of the insulin-like growth factor (IGF)-signaling axis is frequently observed in human hepatocellular carcinoma (HCC). Especially the over-expression of the fetal growth factor IGF- II, IGF- I receptor (IGF-IR), and cytoplasmic downstream effectors such as insulin-receptor substrates (IRS) contribute to proliferation, anti-apoptosis, and invasive behavior. This review focuses on the relevant alterations in this signaling pathway and independent *in vivo* models that support the central role IGF- II signaling during HCC development and progression. Since this pathway has become the center of interest as a target for potential anti-cancer therapy in many types of malignancies, various experimental strategies have been developed, including neutralizing antibodies and selective receptor kinase inhibitors, with respect to the specific and efficient reduction of oncogenic IGF- II /IGF-IR-signaling.

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Key words: Hepatocellular carcinoma; Insulin-like growth factor- II ; Insulin-like growth factor-I receptor; Insulin receptor substrate; Mouse models; Therapy

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INTRODUCTION

Human hepatocellular carcinoma (HCC) is considered the fifth most frequent malignancy worldwide and the third most common cause for cancer mortality with an increasing incidence in Asia and Africa, but also in industrial countries^[1]. In more than 80% of cases, a well-defined etiology such as viral infection with hepatitis B- and C-viruses (HBV and HCV), aflatoxin B1 intoxication, chronic alcohol abuse, or hereditary diseases is associated with its development (Figure 1); however, clinical diagnosis of HCC is difficult due to the lack of reliable serum markers. Moreover, the therapeutic options for HCC patients are sobering due to the high angioinvasive capacity of the tumor.

Although the underlying molecular mechanisms responsible for the development and progression of HCC have not been completely delineated, it has become clear that aberrant activation of growth factor signaling pathways is a pivotal event in hepatocarcinogenesis. Besides the hepatocyte growth factor (HGF)/MET, Wingless (Wnt/frizzled/ β -catenin), transforming growth factor α (TGF α)/EGF-R, and transforming growth factor β (TGF β)/T β R-signaling, dysregulation of the evolutionary highly conserved insulin-like growth factor (IGF) pathway is critically involved in proliferation and anti-apoptosis of HCC cells associated with uncontrolled tumor growth and chemoresistance^[2]. In fact, based on its central regulatory position in tumor cell homeostasis, this signaling axis is considered a promising therapeutic anti-cancer target in many human malignancies. This review focuses on the molecular changes of IGF-signaling detected in human HCC, animal model systems that underline the central role of IGF- II -signaling in hepatocarcinogenesis, as well as resulting therapeutic strategies for the treatment of human liver cancer.

COMPOSITION OF THE IGF-PATHWAY

The key molecules in this pathway are the ligands IGF- I and IGF- II, IGF-binding proteins (IGFBP1-6), membrane-associated receptors [IGF- I receptor (IGF-IR), mannose-6-phosphate receptor/IGF- II receptor (IGF- II R)], and insulin receptor substrates (IRS-1-6).

IGF- I and IGF- II are small, secreted molecules that are predominantly produced by the liver and which stimu-

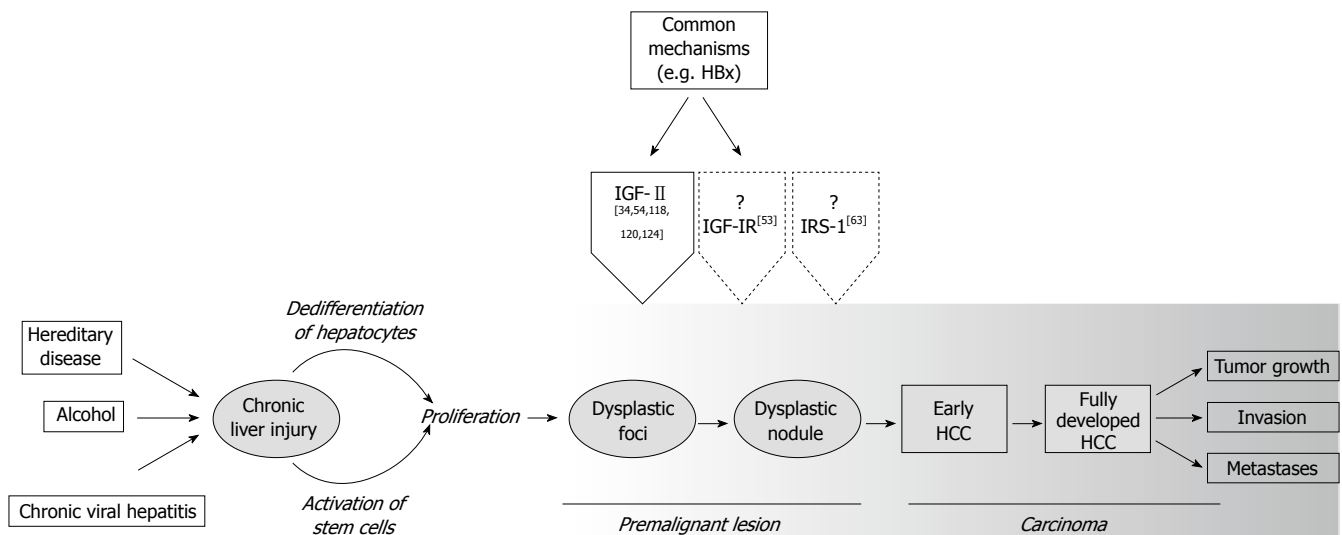


Figure 1 Schematic representation of human hepatocarcinogenesis. Human HCC usually develops on the background of a chronic liver disease (e.g. hepatitis, alcoholic liver disease, hemochromatosis). Dysplastic foci and dysplastic nodules are regarded as premalignant lesions preceding the development of HCC. In addition, “early” HCCs (< 2 cm, highly differentiated, non-invasive) are distinguished from fully developed HCCs (fast growing, invasive). However, human hepatocarcinogenesis represents a developmental continuum where a clear cut classification of a given lesion is often impossible. Increasing evidence suggests that aberrant IGF- II expression represents an early event in hepatocarcinogenesis; however, comparable data are currently not available for IGF-IR, and IRS. Nevertheless, reactivation of the IGF- II /IGF-IR signaling pathway seems to be a progression step in human liver cancer.

late different cell types in both an autocrine and paracrine manner. These factors display differing expression kinetics as the expression of IGF- II declines while the bioavailability of IGF- I increases shortly after birth. Besides the transcriptional regulation (e.g. genomic imprinting of the *igf- II* gene promotor), ligand bioavailability is further influenced by the presence of IGF-BPs in tissues and serum^[3]. Secreted IGF-BPs bind extracellular IGFs with affinities comparable to IGF-IR and therefore modulate ligand bioactivity. For instance, 70% of IGF- II is bound to IGF-BP-3, which is the most abundant BP in serum^[4]; however, depending on the cellular context, both inhibitory as well as stimulatory effects of IGF-BPs on IGF-signaling have been described. All IGF-BPs are substrates for proteases and their bioavailability/bioactivity is regulated by limited proteolytic cleavage with an impact on IGF-dependent physiological processes^[5]. However, IGF-independent biological effects under pathophysiological conditions have also been described for several IGF-BPs^[6].

The signaling of IGF- I and IGF- II is mediated by IGF-IR, a heterotetrameric protein (two α - and β -chains), which consists of an extracellular ligand binding site and an intracellular tyrosine kinase domain. IGF-IR binds IGF- I with 15- to 20-fold higher affinity than IGF- II^[7]. Ligand binding and receptor tyrosine kinase (RTK)-dependent phosphorylation of intracellular substrates such as IRS and Src homology collagen (Shc) then lead to the activation of the phosphatidylinositol 3-kinase (PI3-kinase)/protein kinase B (PKB/AKT)-axis and the Ras/mitogen activated protein kinase (MAPK)-pathway^[8]. IRS proteins are a family of six (IRS-1 to IRS-6) related adaptors that integrate and coordinate signaling of the insulin receptor (IR) and also the IGF-IR. They are responsible for most of the biological activities of IGF-IR^[9].

In addition, IGF- II (but not IGF- I) efficiently binds and activates a distinct isoform of the insulin receptor lacking exon 11 (IR-A)^[10-12]. IR and IGF-IR are highly homologous RTKs (up to 84% in the tyrosine kinase domain and 100% at the ATP-binding site)^[13], but there are substantial functional differences between both molecules: while both receptors exert metabolic effects, IGF-IR is anti-apoptotic, mitogenic, and it facilitates a malignant phenotype^[14]. However, IR-A plays a central role not only in metabolic processes, but also in IGF- II-induced migration in cells lacking IGF-IR^[11]. These different biological effects are possibly based on ligand/receptor abundance, protein turnover or currently undiscovered peculiarities of the distinct signaling axes. In addition, recent findings show that structural features in the domain governing ligand specificity do distinguish IGF-IR from IR^[15].

In contrast, IGF- II R which is structurally unrelated to IGF-IR, does not exhibit cytoplasmic kinase activity^[16]. Although this receptor does not directly contribute to IGF-signaling, it regulates IGF- II turnover and bioavailability through receptor-mediated endocytosis and subsequent degradation^[17]. IGF- I and insulin cross-react very weakly with IGF- II R and therefore are not regulated by its (inhibitory) activity^[18].

IGF-SIGNALING IN HEPATOCARCINOGENESIS

Alterations in the IGF-signaling pathway have been described in several adult and pediatric human tumors such as Wilms tumors^[19], as well as colon^[20,21], lung^[22], breast^[23,24], and prostate cancer^[25]. The reactivation of IGF-

signaling in HCC predominantly occurs at the level of IGF-II expression, which is secreted by the tumor cells themselves, which is suggestive of autocrine mechanisms of stimulation^[26,27]. This growth factor is highly expressed in the fetal liver and early after birth, but its expression is strongly reduced in adulthood in humans, mice and rats^[28-30]. Several studies have shown elevated expression levels for IGF-II in preneoplastic lesions (Dysplastic Nodules) and very high levels in HCC (Table 1, Figure 1), which is mainly based on aberrant activation of the epigenetically regulated *igf-II* promoters P1-P4^[31]. Indeed, HCCs showing high level of expression of IGF-II exhibit reconstitution of the fetal type transcription pattern due to a loss of promotor-specific imprinting and hypomethylation^[26,32-35]. Furthermore, viral proteins have been reported to facilitate IGF-II overexpression in HBV- and HCV-associated HCCs. For example, the HBV-derived HBx protein and the HCV-derived core gene product induce IGF-II expression through interaction with transcription factors activity such as Sp1 and Egr1^[36,37]. In addition, the inactivation of tumor suppressor genes such as p53 by aflatoxin-induced mutations in codon 249 increases IGF-II expression through the formation of transcriptional complexes^[38].

Besides the direct transcriptional induction of IGF-II expression, additional mechanisms may contribute to elevated IGF-II bioavailability in HCC cells. Firstly, reduced levels of IGFBP-1, -2, -3, and -4 in HCCs were found to be associated with IGF overexpression^[39,40]. These IGFBP-based effects on IGF-concentration may be even more complex, since a reduced degradation of IGFBPs by matrix-metalloproteinases (MMPs) was regulated by tissue inhibitors of MMPs (TIMPs). The regulation of TIMP-1, which is repressed in many HCCs, is associated with changes in IGF-II abundance^[41,42]. Secondly, the downregulation or inactivation of IGF-II R theoretically leads to increased concentrations of IGF-II based on insufficient internalization and degradation. Here, the reduced expression of IGF-II R, the loss of heterozygosity (LOH) at the *igf-II* *r* gene locus, homozygous deletions, and missense mutations with an impact on ligand binding have been described with respect to HCCs^[43-49]. However, other studies did not detect any genetic alterations at the *igf-II* *r* locus, which may be due to methodological and population-based differences^[50-52]. Moreover, few studies described elevated IGF-II R levels in HCCs^[53,54]. Independent of the underlying molecular mechanism, IGF-II overexpression denominates a group of HCCs with fewer tumor infiltrating lymphocytes, a lower apoptosis rate^[55] and extrahepatic metastasis^[56]. Thus, serum IGF-II availability was proposed as a tumor marker discriminating HCC from cirrhosis^[57].

IGF-I - and IGF-II-mediated signaling may occur through IGF-IR and IR holoreceptor dimers as well as through IGF-IR/IR hemireceptor complexes^[58,59]. Particularly IGF-II has been shown to efficiently activate both IGF-IR and IR-A. However, our own results suggested that the presence of IR was not essential for IGF-II-mediated oncogenic properties in liver tumor cells, since efficient siRNA-dependent inhibition of IR (all isoforms) did not lead to changes in proliferation, apoptosis, or migration in HCC cells (unpublished data). Therefore, in HCC cells IGF-IR is the relevant receptor for protumorigenic IGF-II

Table 1 Expression of IGF-(II) signaling axis constituents in human HCC

Signaling constituent	Dysregulation (%)
IGF-II	9.2 ^[117]
	22.5 ^[26]
	25.6-60 ^[118]
	66.7 ^[54]
	40 ^[119]
	100 ^[120]
	50 ^[121]
IGF-IR	14 ^[55]
	7-78 ^[117]
IRS-1	40 ^[53]
	46.7 ^[53]
IRS-2	100 ^[122]
	53.3 ^[53]
IRS-4	86 ^[123]
	46.7 ^[53]

signaling. This finding is supported by the fact that IGF-IR is highly expressed in many human malignancies and that only IGF-IR-signaling is crucial for oncogenic transformation and tumor cell survival^[60]. Indeed, while IGF-IR levels were constitutively low in normal hepatocytes, IGF-IR was overexpressed in HCC and HCC cell lines (Table 1). Just as it was observed for elevated IGF-II expression, viral-based molecular mechanisms and mutational inactivation of tumor suppressor genes caused IGF-IR overexpression: HBV-derived HBx protein as well as p53 mutations in codon 249 induce IGF-IR^[61,62], suggesting that these protumorigenic events modulate several IGF-pathway constituents such as IGF-II and IGF-IR to reach maximal (oncogenic) signaling efficiency.

Lastly, IRS-1, -2, and -4 are overexpressed in most HCCs (Table 1). So far, most analyses are reported for IRS-1, showing that elevated IRS-1 levels mediate anti-apoptosis^[63], tumor cell growth^[64], and mitosis^[65]. Further, it has been found that the HCV-derived core protein reduced IRS-1 expression in HCC cell lines^[66]. To our knowledge, no molecular mechanisms responsible for the elevated IRS-1 expression (e.g. other viral proteins) have been described so far. Whether other IRS family members serve identical functions in HCC cells has not yet been analyzed.

In summary, several lines of evidence suggest a 'multi-hit' model for the oncogenic activation of IGF-II signaling in HCC. Firstly, the sum of protumorigenic events detected in HCCs (e.g. increased IGF-II, IGF-IR, and IRS bioavailability) indicates the potential for multiple hits in one single tumor. Secondly, viral proteins and the inactivation of tumor suppressor genes induce several IGF-II pathway constituents. Although increased bioavailability of IGF-II appears to be the dominant mechanism in human hepatocarcinogenesis, many hits in this pathway may be necessary to obtain full malignant competence.

ANIMAL MODELS

The pivotal oncogenic function of IGF-II-signaling

in hepatocarcinogenesis is supported by several animal models. Transgenic mice expressing IGF-II (20-30-fold increased levels in serum) develop hypoglycemia and many types of malignancies, which are most frequently HCC^[67]. In contrast, overexpression of IRS-1 is associated with increased DNA-synthesis, but liver tumor development was not detected^[68]. In knockout model systems the disruption of the *igf-II r* gene leads to elevated IGF-II levels; but since these animals exhibit lethal organ abnormalities (e.g. organomegaly), no further studies concerning liver tumor development have been carried out^[69-71].

In addition to these IGF-pathway-specific transgenic and knockout animals, additional models, initially not intended for the examination of the IGF-axis, supported the functional relevance of especially dysregulated IGF-II in hepatocarcinogenesis. Both mice with liver-directed expression of SV40T-Ag or HBV presurface gene products (preS1 and preS2) developed HCCs, which is associated with a high level of IGF-II expression^[72]. Moreover, transgenic mice overexpressing the woodchuck hepatitis virus/c-MYC^[73], c-MYC^[74], and TGF α ^[75] developed HCCs accompanied by elevated IGF-II expression in the tumors. Equally, liver tumors in p53-null animals exhibited increased amounts of IGF-II as compared to normal littermates after delivery of polyoma virus middle T antigen (PyMT)^[76].

Cross-breeding experiments underlined the importance of IGF-II-signaling in hepatocarcinogenesis. Interbreeding of IGF-II knock-out mice with SV40T-Ag animals resulted in a reduced frequency (up to 15-fold) and size of liver tumors as compared to animals only expressing the oncogene^[77], suggesting an important role of IGF-II-signaling in tumor progression. This anti-tumorigenic effect for IGF-II-deficiency in tumor models was supported by similar results in animals expressing SV40T-Ag in Langerhans cells showing widely identical results^[30]. In a more indirect approach, TIMP1 overexpression reduced IGF-II-driven HCC development in SV40T-Ag transgenic animals based on reduced tumor cell proliferation and vascularization^[41,78,79]. However, it is also noteworthy that mice expressing the c-MYC oncogene and which are deficient for IGF-IR only showed a marginally reduced HCC incidence compared to animals expressing the oncogene alone^[74].

The functional connection between the viral infection of hepatocytes and IGF-II abundance was supported by studies utilizing the woodchuck model system. After woodchuck hepatitis virus (WHV) infection, a high level of IGF-II expression was detected in precancerous woodchuck liver and in up to 45% of HCCs, which correlates with repressed viral DNA replication and n-MYC expression in early precancerous lesions^[34,80]. Further studies revealed that IGF-II availability protected from n-MYC-induced apoptosis especially under serum-free conditions^[81]. Therefore, the selection for cells with high IGF-II levels may rescue a more unfavorable tumor phenotype and therefore promote tumor progression. Lastly, a reactivation of IGF-II expression in experimentally induced liver tumors using different chemical substances (3'-Me-DAB, 2-AAF, DENA) has been described in rats^[82-84].

These data clearly show that IGF-II overexpression and intactness of the IGF-II/IGF-IR pathway is also a common event in murine liver tumor development, independent of the underlying molecular mechanisms (e.g. oncogene activity, regeneration processes, chemically induced carcinogenesis)^[72].

THERAPY

IGF-II is highly expressed during prenatal development and early after birth but levels rapidly decline in adulthood^[28,29]. Since IGF-II signaling is frequently reactivated in human hepatocarcinogenesis, inhibition of this pathway unlikely affects normal liver function under physiological conditions and therefore represents a favorable therapeutic strategy. Several techniques have been developed to modulate the activity of IGF-(II) signaling in different tumor cell types^[85]. Many approaches, such as neoexpression of dominant-negative receptor mutants (dnIGF-IR) or transfection of IGF-IR-specific antisense oligodeoxynucleotides, attained convincing inhibitory effects on IGF/IGF-IR signaling *in vitro* and *in vivo*^[85]. However, neutralizing antibodies binding IGF-IR and IGF-IR-specific small inhibitory molecules are currently the most promising therapeutic and clinically relevant approaches^[60].

Neutralizing antibodies

Recently, numerous blocking antibodies recognizing different membrane-bound RTKs such as EGF-R/HER1 (Cetuximab/Erbitux) and HER2 (Trastuzumab/Herceptin) have been developed^[86]. Besides IGF-II-binding antibodies that physically inhibit ligand/receptor interaction^[87,88], many neutralizing antibodies specific for IGF-IR have been described such as alpha-IR3^[89], mAb391^[90], scFv-FC^[91], CP-751,871^[92], IMC-A12^[93], 7H2HM^[94], EM164^[95], h7C10^[96], 4G11^[97], 19D12^[98], R1507^[60], AMG479^[60], and 19D12^[60]. Reduced IGF/IGF-IR signaling is presumably based upon lysosome-dependent degradation of IGF-IR^[90,91]. Since proteasome inhibitors (e.g., Brefeldin) as well as protein synthesis inhibitors (e.g., cyclohexamide) did not affect antibody-dependent downregulation of the receptor^[90,91], it has been speculated that anti-IGF-IR antibodies hampered steady-state protein turnover based on endosomal accumulation of antibody/receptor complexes^[99]. Although the anti-tumor effects of these antibodies were tested for several different cell types in preclinical studies, no comprehensive analyses regarding the anti-tumorigenic impact on HCC cells have been published to date. However, it is noteworthy that for other tumor entities, clinical trials for antibodies targeting IGF-IR have been launched such as CP-751, 871 (Pfizer), IMC-A12 (ImClone Systems), R1507 (Roche), and AMG479 (Amgen)^[60].

Tyrosine kinase inhibitors

In addition to neutralizing antibodies, small molecule inhibitors targeting RTKs such as EGF-R/HER1 (Gefitinib/Iressa), BCR/ABL fusion product (Glivec/Imatinib), or cellular kinases (the multi-kinase inhibitor Sorafenib/Nexavar recognizing VEGF-R, PDGF-R, c-kit, Raf, and

RET) have been developed. Since IGF-IR and the IR are structurally related, highly specific IGF-IR inhibitors are necessary to prevent diabetogenic effects in patients. Published IGF-IR-selective RTK-inhibitors are tyrphostins (AG538^[100,101], AG1024^[102], AG1034^[102]), cyclolignans (picropodophyllin^[103,104]), 6-5 ring-fused compounds^[105], pyrrole derivatives (NVP-AEW541^[106,107], NVP-ADW742^[108,109]), PQIP^[110], BMS536924^[111], and BMS-554417^[112]. Anti-tumorigenic effects of some inhibitors on HCC cells have been demonstrated. The application of NVP-AEW541^[113] and picropodophyllin (Nussbaum *et al*, unpublished data) was shown to reduce tumor cell proliferation and increase apoptosis. Equally, IGF-II induced tumor cell motility was reduced by picropodophyllin (Nussbaum *et al*, unpublished data). In addition, the inhibition of IGF-IR-signaling by a combination of AG1024 and EGF-R-signaling by RTK-inhibitors or blocking antibodies synergistically reduced tumor growth^[114,115]. However, NVP-ADW742 affects the viability of hepatocytes in a concentration-dependent manner. This RTK-inhibitor potentiated bile acid-induced cell death in normal hepatocytes, suggesting liver toxicity in patients with aberrant bile flow^[116]. Because IGF-IR signaling is almost absent in normal hepatocytes, it is questionable whether these effects were IGF-dependent or independent. Thus, the effects of IGF-IR-specific inhibition on normal and diseased liver have to be analyzed carefully.

Although the anti-tumorigenic effects of IGF-IR-specific small molecules have been analyzed in numerous tumor cell types in preclinical setups^[60], to our knowledge, no clinical trials have been initiated to date.

CONCLUSION

Several components of the IGF-signaling axis, such as IGF-II, IGF-IR and IRS, are frequently dysregulated in human hepatocarcinogenesis. The oncogenic reactivation of IGF-II-signaling has been verified in several *in vivo* models and supports the therapeutic relevance of this pathway. However, aberrant growth factor bioactivity involved in tumor development cannot be understood in a mono-dimensional manner since an intense cross-talk between IGF-IR signaling and other oncogenic pathways have been described^[2]. Indeed, first functional studies revealed the necessity for multi-modal approaches for optimal anti-tumorigenic results and dose reduction. Therefore, it is questionable whether the highest specificity is the 'gold standard' for efficient treatment of malignancies, especially with respect to the development of (IGF-IR specific) RTK inhibitors. Thus, inhibitors targeting IGF-IR and other RTKs or combinations of different specific substances targeting distinct pathways might be attractive therapeutic approaches in the future.

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Activins and activin antagonists in hepatocellular carcinoma

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Abstract

In many parts of the world hepatocellular carcinoma (HCC) is among the leading causes of cancer-related mortality but the underlying molecular pathology is still insufficiently understood. There is increasing evidence that activins, which are members of the transforming growth factor β (TGF β) superfamily of growth and differentiation factors, could play important roles in liver carcinogenesis. Activins are disulphide-linked homo- or heterodimers formed from four different β subunits termed β A, β B, β C, and β E, respectively. Activin A, the dimer of two β A subunits, is critically involved in the regulation of cell growth, apoptosis, and tissue architecture in the liver, while the hepatic function of other activins is largely unexplored so far. Negative regulators of activin signals include antagonists in the extracellular space like the binding proteins follistatin and FLRG, and at the cell membrane antagonistic co-receptors like Cripto or BAMBI. Additionally, in the intracellular space inhibitory Smads can modulate and control activin activity. Accumulating data suggest that deregulation of activin signals contributes to pathologic conditions such as chronic inflammation, fibrosis and development of cancer. The current article reviews the alterations in components of the activin signaling pathway that have been observed in HCC and discusses their potential significance for liver tumorigenesis.

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Key words: Hepatocellular carcinoma; Activin; Follistatin; Transforming growth factor β

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the predominant form of primary malignancy of the liver and accounts for more than half a million deaths per year^[1]. In some geographical regions it is the most prevalent form of malignancy and the most common cause of death from cancer^[2] making its containment a top priority. Chronic infection with the hepatitis B or C virus (HBV, HCV), dietary exposure to the hepatocarcinogen aflatoxin B1 (AFB1), ethanol abuse, and obesity are among the main risk factors for liver cancer^[3]. Despite recent advances, the molecular pathology of the disease is not well understood and the therapeutic possibilities are largely limited to surgical procedures including resection, liver transplantation, or local tumor ablation^[4]. A consistent pattern of changes comparable to the sequential mutations in tumor suppressor genes and oncogenes, like the one identified in colon carcinogenesis during adenoma to carcinoma progression^[5,6], has not been defined for HCC. Nevertheless, multiple genetic alterations including mutations of p53, inactivation of the Rb pathway, and activation of the Wnt/ β -catenin pathway have been linked to HCC development and progression^[3,7]. In addition deregulated expression of growth factors and their cognate receptors has been described in HCC for both, positive regulators of hepatocyte growth, such as insulin-like growth factor 2 (IGF-2), hepatocyte growth factor (HGF), and transforming growth factor α (TGF α), as well as for negative regulators like TGF β ^[8].

In recent years activins, a subgroup of the TGF β family of growth, differentiation, and death factors which share part of their signaling mechanisms with TGF β , have gained attention with respect to their role in tumor development in several organs^[9]. Activin subunits, their receptors and several antagonistic proteins are expressed in the normal liver. Deregulation of this balanced expression

appears to contribute to hepatic dysfunctions like impaired regeneration, fibrogenesis and tumorigenesis^[10]. The current knowledge about the role of activins and activin antagonists in HCC is discussed in this review.

ACTIVINS-STRUCTURE AND SIGNALING

Activins are secreted polypeptides and represent a subgroup of the TGF β superfamily of growth and differentiation factors. Additional members of this superfamily include TGF β 1-3, bone morphogenetic proteins (BMPs), growth and differentiation factors (GDFs), myostatin, Mullerian inhibiting substance (MIS), nodal and several others^[10,11]. Activins are homo- or heterodimers composed of four different β subunits (β A, β B, β C, β E), each encoded by a single gene. The β subunits can either form activins by dimerization with a second β subunit, or alternatively can form inhibins by dimerizing with a single α subunit encoded in mammalian genomes^[12]. Activin terminology is dependent on the dimer configuration with a single letter designating homodimers (activins A, B, C, and E) and two letters designating heterodimers according to their subunit composition (activins AB, AC, AE, BC *etc.*). With respect to tissue expression, transcripts of the β A and β B subunits were found to be detectable in almost all tissues analyzed with especially high expression in reproductive organs^[13,14]. The β C and β E subunits, in contrast, are predominantly expressed in the liver and at lower levels in a limited number of additional organs^[14-18].

Activin β subunits are synthesized as precursor molecules with 350-426 amino acids and molecular weights between 38 kDa and 50 kDa^[19]. The prodomains are removed in the ER and in the early Golgi by members of the protease family of subtilase-like pro-protein convertases (SPC)^[20] to release mature peptides with either 115 (β B, β E) or 116 (β A, β C) amino acids. The amino acid sequences of the mature peptides are approximately 50% conserved among the four human β subunits, whereas the sequence homology in the prodomain is only about 20%. Analysis of the phylogenetic relationship of the mature human peptides groups together β A and β B on the one and β C and β E on the other hand^[21].

Like other members of the TGF β family, the activin β subunits contain nine conserved cysteines in the mature peptides. The sixth is used for dimerization, whereas the other eight form intramolecular disulfide bonds which determine the three-dimensional structure of the peptides^[22]. While all cysteines in the mature chain of activin β A are necessary for biosynthesis of activin A dimers or for their full biological activity, four additional cysteines in the prodomain are dispensable for dimerization and secretion. Protein folding and dimerization take place in the lumen of the ER and are catalyzed by members of the protein disulfide isomerase (PDI) family^[23,24]. Unlike TGF β , which is secreted as a latent complex consisting of the TGF β homodimer, its prodomain (also termed latency-associated propeptide, LAP), and the latent TGF β binding protein (LTBP)^[25], activins are secreted as dimers of the mature peptides and need no further processing in the extracellular space to gain bioactivity. Activin A signals are transduced *via*

two types of single-pass transmembrane serine threonine kinase receptors, termed activin receptors type I and type II^[26]. Activin A first binds to the type II receptors which in turn recruit and phosphorylate the type I receptors^[27]. Two type II receptors for activin A (ActR-II (A) or ACVR2 (A) and ActR-II B or ACVR2B) have been identified. The main type I receptor for activin A is ALK (Activin Receptor-Like kinase) 4, also designated as ActR-IB or ACVR1B, whereas activins B and AB have a preference for ALK 7 (ACVR1C) as type I receptor^[28]. Receptors for activins containing β C or β E subunits have not been identified so far. Activin C, however, did not compete with activin A for receptor binding^[29] and a chimeric activin construct in which the receptor binding sequence (amino acids 46-78) of β A was replaced by the corresponding region of β C retained type II receptor binding but was unable to recruit the type I receptor ALK 4^[30].

Inhibins have been shown to form a complex with type II receptors via their β subunits and with betaglycan also known as TGF β type III receptor. The α subunit, however, is unable to bind type I receptors and consequently activin receptor signaling is inhibited^[31,32]. There is in general a considerable degree of promiscuity in receptor usage by different TGF β superfamily members. In addition to activin A, for instance, myostatin, and several BMPs were shown to signal *via* ActR-II B^[33].

Phosphorylated TGF β family receptors recruit intracellular mediators called Smads, which transduce activin signals to the nucleus^[26]. Smads can be divided into receptor Smads (Smads 1, 2, 3, 5 and 8), a common mediator Smad (Smad 4) and inhibitory Smads (Smads 6 and 7). Activin A receptors, as well as TGF β receptors, recruit and phosphorylate the receptor Smads 2 and 3, whereas receptor Smads 1, 5, and 8 are recruited by BMP receptors but not activin receptors^[34]. Recent evidence suggests that-similar to TGF β -additional Smad-independent signaling pathways may contribute to activin A signaling, as for instance, RhoA, MEKK1, JNK, and p38 were found to be involved in activin-induced cytoskeleton reorganization and cell migration in keratinocytes and in promoter activation of the transcription factor Pit-1 in pituitary lactotrope cells^[35,36].

Activin signals are tightly regulated on the one hand by a spatially and temporally restricted production of activin subunits and on the other hand by the expression of several extra- as well as intracellular antagonists of activin signaling. An overview of activin-mediated signaling events and the corresponding interaction points with endogenous activin antagonists is presented in Figure 1.

ACTIVIN SUBUNITS AND ACTIVIN ANTAGONISTS IN LIVER CANCER

Activin β A

Activin A, the homodimer of two β A subunits, is by far the most extensively investigated activin. Multiple biological functions of activin A in a variety of cells and tissues have been described. Activin A has been implicated for instance in mesoderm induction^[37], stem cell biology^[38], reproductive biology^[39], erythroid differentiation^[40], systemic inflammation^[41], cell death induction^[42], wound healing^[43],

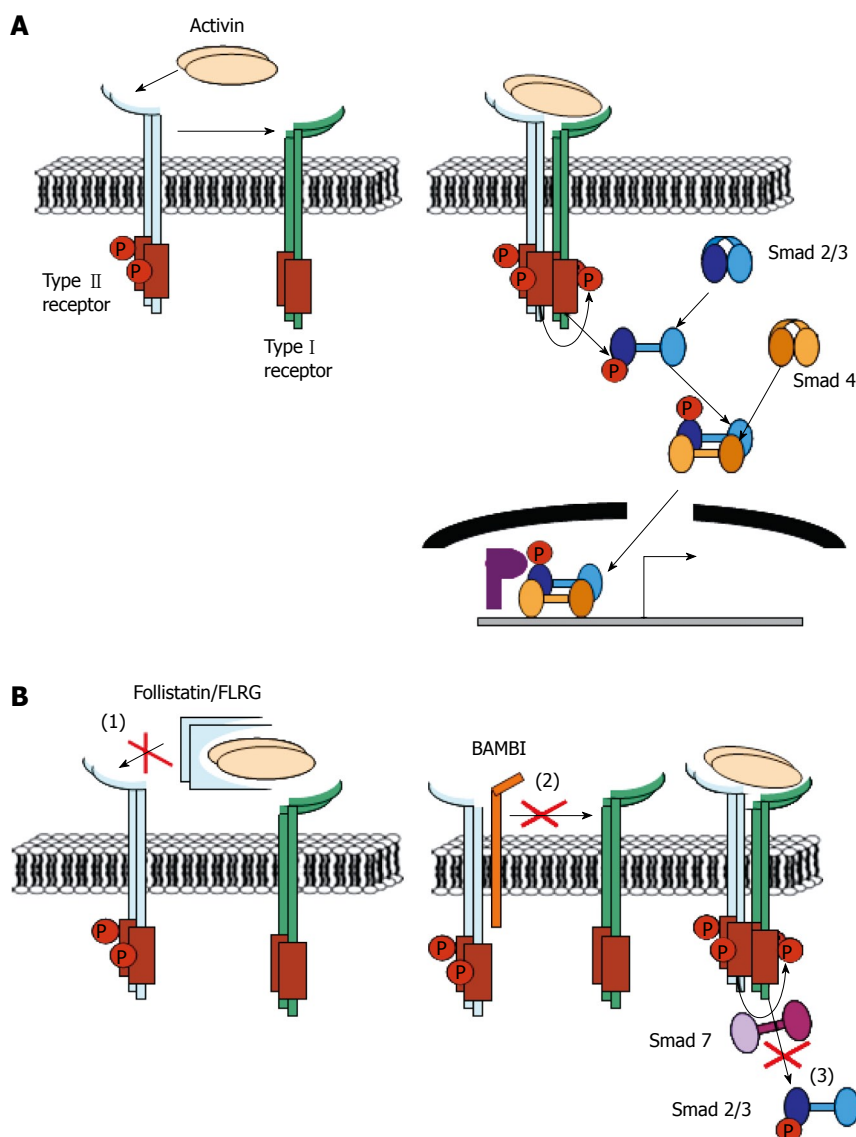


Figure 1 Graphic representation of activin signaling and interaction points with activin antagonists. **A:** Activin dimers first bind the type II activin receptors, which then recruit and phosphorylate type I receptors. These in turn phosphorylate receptor-activated Smads, which subsequently form a complex with Smad 4 and are translocated to the nucleus, where they regulate the transcription of target genes; **B:** Activin antagonists can block activin signals by: (1) Binding activins in the extracellular space like follistatin or FLRG and thereby blocking their access to activin receptors; (2) Acting as inhibitory co-receptors, which prevent ligand receptor interactions (Cripto) or receptor dimerization (BAMBI); (3) Competing with receptor-activated Smads 2 and 3 for binding sites on activin receptors (Smad 7).

and fibrosis^[44]. Knock-out mice for βA have severe defects in craniofacial development and die shortly after birth^[45]. Concerning the liver, activin A potently inhibits mitogen-induced DNA synthesis and induces apoptosis in hepatocytes *in vivo* and *in vitro*^[46-48]. Activin βA antisense oligonucleotides stimulated cell proliferation in the human hepatoma cell line HLF suggesting a growth inhibitory function of endogenous activin A^[49]. In regenerating liver, activin βA gene expression was reduced at time points when hepatocyte replication took place and was increased at later periods when liver regeneration terminated^[50]. Increased expression of βA at earlier time points after partial hepatectomy, however, has also been described^[51,52]. Besides the effects on DNA synthesis and cell growth, activin A also regulates restoration of liver architecture after partial hepatectomy by stimulating collagen production in hepatic stellate cells (HSC) and tubulogenesis of sinusoidal endothelial cells^[53,54]. Stimulation of HSC may also contribute to liver fibrosis and several investigations have found elevated levels of activin βA in fibrotic and cirrhotic rat livers^[55-58]. Elevated levels of circulating activin A were found in patients suffering from chronic viral hepatitis or alcohol induced liver cirrhosis and in HCC patients^[59-61].

Reduced expression of activin βA transcripts in contrast, was observed in tumor tissue from chemically-induced rat liver tumors and in 5 of 11 HCC specimens^[62]. In addition to a pro-apoptotic effect on the parenchymal cells and a pro-fibrotic effect on HSC, activin A has also been linked to neoangiogenesis *via* stimulation of VEGF expression in human hepatoma cells^[63].

Activin βB

Like activin βA , the βB subunit is expressed in multiple tissues and organs^[13,14]. Despite a considerable overlap in tissue expression and in some biological activities, important differences exist^[64]. Knock-out mice for βB are viable but have defects in eyelid development and female reproduction^[65]. When the coding region of the mature peptide of the βA subunit gene was replaced with the corresponding region of the βB subunit gene the developmental defects of the βA knock-out mice were only partially rescued^[66]. Concerning the liver, the role of the βB subunit is not well characterized. In the normal rat liver the βB subunit was the only activin subunit undetectable by RNase protection assay^[14]. Weak positive immunoreactivity for βB was, however, detected in hepatocytes of normal

rat livers and in connective tissue septa in fibrotic livers when analyzed by immunohistochemistry^[55]. Activin β B mRNA was induced in stellate cells of CCl₄ treated rat livers^[55]. Exposure to the peroxisome proliferator di-n-butyl phthalate led to a transient surge of β B mRNA expression 6 h after treatment in rat livers^[67]. With respect to biological activities, recombinant activins A and AB but not activin B inhibited EGF induced DNA synthesis in primary rat hepatocytes^[68]. In normal human liver the β B transcript is readily detectable by RT-PCR (M.G. unpublished observation), but no data with regard to expression changes of the β B subunit in liver tumors compared to normal liver have been reported yet.

Activin β C

The activin β C subunit was cloned from liver cDNA and demonstrated to be predominantly expressed in hepatocytes by Northern blot analysis and RNase protection assays^[14,18,52,69]. By immunohistochemistry significant activin β C expression has been detected in cells from additional organs including the prostate, ovary, testes, and pituitary gland^[15,70]. Formation of homodimeric activin C as well as heterodimeric activins AC, BC, CE, as well as inhibin C has been demonstrated by ectopic expression of the respective subunits in different cell models^[14,70,71]. After partial hepatectomy a transient down-regulation of activin β C expression was observed by several studies^[50,52,72,73]. A decrease of activin β C expression has also been observed in HepG2 and Hep3B hepatoma cells versus normal liver tissue^[74] and in rat hepatocytes during primary culture with and without EGF treatment^[52]. In contrast, increased activin β C expression was reported in the rat liver during the development of CCl₄ induced cirrhosis^[56,75] and in response to treatment with the peroxisome proliferator bi-n-butyl phthalate^[67]. The functions of the activin β C subunit are controversial. Activin β C knock-out mice developed normally and liver regeneration after partial hepatectomy proceeded similarly in knock-out animals and wild-type littermates^[76]. Ectopic expression of activin β C induced apoptosis in human (HepG2, Hep3B) and rat (H4 IIEC3) hepatoma cells and delayed liver regeneration in mice^[74,77]. In AML12 cells, an immortalized mouse hepatocyte cell line in contrast, and in primary rat hepatocytes, activin β C increased DNA synthesis^[29]. Adenovirus-mediated expression of activin β C accelerated liver regeneration after partial hepatectomy in rats^[78]. A specific association of activin β C immunoreactivity with mitotic hepatocytes was observed in regenerating liver after partial hepatectomy^[50]. It was shown that activin C does not activate activin A-responsive promoters, and it was suggested that the β C subunit regulates the levels of bioactive activin A *via* the formation of signaling-incompetent activin AC heterodimers in PC3 human prostate cancer cells^[79,80]. Data regarding the expression of the β C subunit in human liver tumors are not available yet.

Activin β E

Similar to activin β C, the β E subunit is predominantly expressed in hepatocytes but has also been detected in human heart, testis, peripheral blood leucocytes, placenta, and skeletal muscle^[14,16,21,81]. Formation of homodimeric

activin E as well as heterodimeric activins AE and CE has been demonstrated after ectopic co-expression of the respective subunits^[14,16]. Activin β E mRNA expression was transiently up-regulated after partial hepatectomy or portal vein branch ligation^[73,76] and in response to lipopolysaccharide treatment^[81]. Increased β E expression has also been observed in hepatic fibrosis induced by CCl₄^[75]. Recently, induction of β E expression has been described as a marker for phospholipidosis in HepG2 hepatoma cells^[82]. Similar to β C, β E subunit knock-out mice and double knock-outs lacking both β C and β E expression developed normally and had no defects in liver function^[76]. When ectopically expressed in HepG2 or Hep3B hepatoma cells or in the murine hepatocyte cell line AML12, activin β E reduced cell number and increased apoptosis rates^[74,83]. Transient overexpression of β E by non-viral gene transfer in the mouse liver inhibited regenerative DNA synthesis^[77]. These observations suggest that activin E may have a growth-limiting function similar to activin A, however, the two subunits show a reciprocal pattern with respect to diurnal variations of expression^[10]. In line with a growth-limiting function of activin E, transgenic mice overexpressing β E in the pancreas showed reduced proliferation of pancreatic exocrine cells^[84]. Regarding liver cancer, reduced expression of the β E subunit was found in human HCC specimens as well as in N-nitroso morpholine-induced rat liver tumors^[62,85]. Interestingly, activin β E expression was found to be regulated by the tumor suppressor gene RASSF1A^[86], a gene frequently inactivated by promoter hypermethylation in HCC^[87,88].

Inhibin α

The inhibin α subunit is part of inhibins but not activins and in many biological systems activins and inhibins have antagonistic effects^[89]. Historically activins received their name from the fact that they activated follicle stimulating hormone (FSH) secretion from the pituitary, whereas the previously described inhibins represented the long sought-after gonadal feed-back inhibitor of pituitary FSH secretion^[12]. Knock-out mice for the inhibin α subunit developed gonadal sex-cord stromal tumors suggesting a tumor suppressive function of the inhibin α subunit^[90]. In several human tumor types including some types of ovarian carcinoma and adrenal tumors, in contrast, overexpression of inhibin α has been demonstrated, and inhibins have been used as serum markers for early detection of ovarian germ cell tumors and monitoring of recurrence^[9]. With regard to liver cell growth, treatment with inhibin A *per se* had no effect on DNA synthesis of HepG2 hepatoma cells but antagonized the inhibitory effect of activin A^[91]. In normal and fibrotic rat liver absence of inhibin α subunit immunoreactivity has been reported^[55]. Immunostaining for inhibin α has been used to distinguish adrenal cortical tumors, which are positive in about 70% of cases, from HCC and renal cell carcinoma, which are mostly negative^[92,93].

Follistatin

Follistatin is a secreted, monomeric glycoprotein lacking homology to the TGF β superfamily. The biological

activities described for follistatin, however, seem to depend entirely on its interaction with activins and other members of the TGF β family. Follistatin is expressed in most of the organs, that also express activin^[13,94], and it binds mature secreted activin A with very high affinity (Kd 50-680 pmol/L)^[95-97]. Complex formation with follistatin completely abolished receptor binding of activin A, thus blocking activin signaling^[96,98]. Two follistatin molecules embrace one activin dimer and bury one-third of its residues and its receptor binding sites^[99]. Three major forms of secreted follistatin exist, resulting from alternative splicing and protein processing of a single follistatin gene and containing 288, 303 and 315 amino acids, respectively^[95]. All forms of follistatin contain three homologous follistatin domains^[100] of which the first two, but not the third, are necessary for activin A binding^[97,101]. Follistatin 288 binds to heparan sulfates, whereas this binding is blocked by an acidic tail in follistatin 315^[95]. In addition to binding activins A, B, AB, and E, follistatin was also shown to bind and antagonize myostatin as well as BMPs 2, 4, 6 and 7^[16,102-105]. Follistatin administration by intraportal infusion or adenovirus-mediated overexpression caused DNA synthesis and liver growth in normal rat livers presumably by antagonizing tonic inhibition of liver growth by activin A^[106,107]. Following partial hepatectomy follistatin expression was up-regulated after 24-48 h, the time period in which hepatocyte replication was increased^[50]. Under similar conditions administration of follistatin accelerated liver regeneration but led to impaired restoration of normal tissue architecture and compromised liver function^[108-110]. Administration of exogenous follistatin in CCl₄ treated rats attenuated the formation of liver fibrosis^[111]. These results likely reflect the ability of follistatin to antagonize both growth-inhibitory and pro-fibrotic activities of activin A.

In human liver cancer and also in animal models follistatin expression was increased in about 60% of tumor tissues. Increased follistatin levels were also found in the blood of patients with liver cirrhosis and HCC^[60,62,112]. Administration of follistatin stimulated DNA synthesis in preneoplastic rat hepatocytes in an *ex vivo* system, whereas hepatoma cell lines were unresponsive to exogenous follistatin possibly due to autocrine production of follistatin or other activin antagonists^[62,112-114].

FLRG

Follistatin-related protein, encoded by follistatin-related gene (FLRG), also designated as follistatin-like 3 (FSTL-3) has a high similarity to follistatin and shares its ability to bind TGF β family proteins, but contains only two instead of three follistatin domains^[115]. Several other proteins, containing 1-10 follistatin domains, like the extracellular matrix-associated proteins SPARC and agrin, on the other hand were not able to bind TGF β family members^[100,116]. The FLRG gene was originally identified as a target of chromosomal rearrangement in leukemia^[117]. The highest tissue expression of FLRG was found in placenta, whereas highest follistatin expression was found in ovary, testis, and pituitary^[115,118]. In HepG2 hepatoma cells, expression of both FLRG and follistatin was induced in response to activin A treatment suggesting that they participate

in a feedback loop to restrict activin A signals^[119]. FLRG mRNA is up-regulated in rat livers in response to a necrogenic dose of CCl₄ (M.G. unpublished observation) but otherwise the role of FLRG in liver regeneration has not been characterized. Elevated expression of FLRG was found in chemically induced rat liver tumors and H4 II E rat hepatoma cells but not in human liver tumor specimens^[62] indicating species-specific differences with respect to FLRG regulation or differences between liver tumors of different etiologies.

Activin receptors

The type II activin receptors ActR-II (A) and ActR-II B and the type I activin receptors ALK4 and ALK7 are expressed in multiple cell types and tissues including the liver. Adenovirus-mediated overexpression of a dominant-negative type II activin receptor caused DNA synthesis and liver growth in normal rat livers^[120]. During liver regeneration after partial hepatectomy, no change of ActR II was observed while ActR II B was transiently decreased^[50]. During CCl₄ induced rat liver cirrhosis, ActR II A was reduced after 5 wk but returned to control levels after 10 wk^[56]. Ectopic overexpression of ActR-IB (ALK4) and ActR- II B or of ALK7 induced apoptosis in hepatoma cells^[121,122]. In HCC tissue specimens, expression of activin receptors (ActR-I, ActR-IB, ActR- II, and ActR- II B) was demonstrated by immunohistochemistry^[63]. Inactivating mutations of activin receptors have been found in microsatellite instable colon cancer, pancreatic cancer and prostate cancer, but have not been investigated in HCC so far^[123-126].

Regulators of activin receptor activity

Several membrane-associated proteins exist which regulate activin-induced receptor activation. Cripto/TDGF1 is a member of the EGF-CFC (epidermal growth factor-Cripto/frl/cryptic) family of growth factor-like molecules. This secreted protein can attach to the outer cell membrane via a glycosylphosphatidylinositol anchor and functions as a co-receptor for nodal signaling during embryogenesis. Cripto has been found overexpressed in high percentages of several human malignancies including breast, pancreas, lung, colon and bladder cancer^[127]. Cripto inhibits ligand receptor interactions of activins and TGF β ^[128-130] and this has been suggested to contribute to its pro-tumorigenic activity. However, an additional activin receptor-independent signaling pathway for Cripto involving Glypican-1 and c-Src has also been described^[127]. Expression of a shorter Cripto variant was observed in colon cancer including liver metastases, as well as in colon cancer and hepatoma cell lines^[131,132]. Expression of this short variant is driven by Wnt signaling which is frequently constitutively activated in colon cancer and HCC. Based on these findings, a more extensive investigation on the role of Cripto in HCC is certainly warranted.

BAMBI (bone morphogenetic protein and activin membrane-bound inhibitor) also known as nma (non metastatic gene A) is a pseudoreceptor related to the type I receptors of the TGF β family. It lacks an intracellular kinase domain and inhibits activin A, TGF β , and BMP signaling by stably associating with TGF β family

Table 1 Overview of described alterations of activins and activin antagonists in HCC and HCC-derived cell lines

Activin subunits and activin antagonists	Proposed function in activin signaling	Alterations observed in HCC and hepatoma cells
Activin β A subunit	Activates activin receptors	Increased activin A in circulation of HCC patients ^[60,61] Decreased expression in rat and human liver tumors ^[62] Loss of expression in hepatoma cells ^[74,114]
Activin β E subunit	Induces apoptosis by as yet undefined mechanisms	Decreased expression in rat and human liver tumors ^[62,85]
Follistatin	Binds activins and blocks their interaction with receptors	Increased in circulation of HCC patients ^[60] Increased expression in human mouse and rat liver tumors ^[62,112] Expressed in hepatoma cells ^[112,114]
FLRG	Binds activins and blocks their interaction with receptors	Increased in rat but decreased in human liver tumors ^[62]
BAMBI	Binds TGF β -family type II receptors and blocks type I receptor activation	Increased in HCC and colon cancer ^[135]
Cripto	Blocks interaction of activins (and TGF β) with their receptors	Overexpressed in hepatoma cells ^[132]
Smad 7	Inhibits activation of Smads by activin (and TGF β) receptors	Increased expression in HCC ^[146,147]

receptors^[133]. A recent study links LPS/Toll-like receptor 4-induced downregulation of BAMBI in hepatic stellate cells to hepatic fibrosis^[134]. In contrast, elevated BAMBI expression driven by the Wnt/ β -catenin pathway was found in HCC and CRC specimens^[135].

ARIPS 1 and 2 (activin receptor-interacting proteins) are PDZ (PSD-95/Discs-large/ZO-1) protein-protein interaction domain-containing proteins that were described to interact with type II activin receptors and inhibit or augment activin signaling, depending on the isoforms expressed^[136-138]. ARIP 2 was recently shown to be induced by activin A in the mouse hepatoma cell line Hepa1-6 and to decrease activin-mediated collagen IV expression, suggesting that it participates in a negative feedback regulation of activin-induced liver fibrosis^[139]. Data with regard to a role of ARIPS in HCC or other tumor types are missing so far.

Intracellular inhibitors of signal transduction

Downstream from activin receptors, signals are transduced by receptor Smad 2 and Smad 3 and the common mediator Smad 4, the same set of Smad proteins also used by TGF β receptors. Mutations of Smad proteins are frequent in pancreatic and colorectal cancer and have also been detected in HCC^[140-142]. Smads 6 and 7 associate with TGF β family receptors but are not phosphorylated and thus inhibit signal transduction^[143,144]. Smad7 has been demonstrated to inhibit activin signaling and to protect hepatocytes from activin A-induced growth inhibition^[145]. Increased expression of Smad7 has been observed in HCC tissue compared to adjacent tissue^[146] and in advanced HCC compared to early HCC or dysplastic nodules^[147]. No mutations of either Smad 6 or Smad 7 were found in 52 HCC samples^[148].

Smurf-type ubiquitin E3 ligases, Smad anchor for receptor activation (SARA), and transcriptional co-activators and co-repressors such as CBP, p300, c-Ski, and SnoN, control Smad activation by TGF β -family receptors or shuttling of activated Smads into the nucleus as well as transcriptional activity of Smad-containing complexes^[42]. Their role in the link between activin signals and liver carcinogenesis has yet to be defined.

In summary, increasing evidence suggests that deregulation of activin signals frequently occurs in and contributes to HCC development and progression. An

overview of alterations in activin subunits and activin antagonists described in liver tumors and hepatoma cells is presented in Table 1.

THERAPEUTIC PERSPECTIVES

Activin signaling is complex. At least three features of the activin signaling cascade contribute to this complexity. First, four activin β and one inhibin α subunit can give rise to multiple homo and heterodimers with different receptor binding capabilities. Secondly, a number of different extracellular activin-binding and receptor-interacting proteins can modulate ligand receptor interactions not only of activins but also of TGF β , BMPs and GDFs. Thirdly, there is a considerable degree of promiscuity with respect to usage of receptors and intracellular signaling molecules between different members of the TGF β superfamily^[149]. For instance, activins and TGF β use different type I and type II receptors but rely on the same Smad proteins for intracellular propagation of their signals. This makes it a difficult task to dissect their specific contribution to biological activities, especially in tissues such as the liver, where both activins and TGF β are expressed. In addition, BAMBI, Cripto and Smad7 have all been shown to interfere with signal transduction of activins as well as of TGF β .

TGF- β 1 has a well recognized dual role in carcinogenesis^[150]. It acts as a tumor suppressor in early stages of hepatocarcinogenesis by inducing apoptosis and eliminating precursor lesions^[151,152]. At a later stage, however, liver tumor cells often become resistant to its proapoptotic effect, and produce large amounts of TGF β themselves^[153]. From the available data on both loss of expression in tumor cells and apoptosis induction^[74,114,154], one would postulate that activin A, and possibly activin E, may have a similar tumor suppressive function in the liver as TGF β . Whether also activins may shift to a pro-tumorigenic function during tumor progression is little explored. For activin A, a contribution to liver fibrosis, enhanced expression of the angiogenic factor VEGF in hepatoma cells, and stimulation of growth and invasiveness of esophageal squamous cell carcinoma cells has been demonstrated^[158,63,155].

Despite all the complexity, however, a general theme

in HCC and in other tumor types seems to be the elevated expression of activin antagonistic proteins in the tumor cells, as observed for follistatin, BAMBI, Cripto, and Smad7^[62,127,135,146,147]. These may serve to block the growth inhibitory and pro-apoptotic activity of activin A on hepatocytes. Similar observations have been made in additional tumor types, for instance for Cripto in multiple epithelial tumors, BAMBI in colon carcinoma, follistatin in melanoma and FLRG in breast cancer^[127,135,156,157].

Consequently, a targeted inhibition of activin antagonists might restore sensitivity to activin-induced growth inhibition and apoptosis, and may thus represent a feasible strategy to inhibit tumor growth. In line with this hypothesis, it has recently been shown that siRNA-mediated silencing of FLRG inhibited breast tumor cell growth *in vitro*, and that monoclonal antibodies to Cripto inhibited growth of testicular and colon cancer cells in xenograft models^[128,156]. Future studies will have to clarify whether such approaches may offer new therapeutic opportunities for combating liver cancer.

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TOPIC HIGHLIGHT

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Current role of ultrasound for the management of hepatocellular carcinoma

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Abstract

Hepatocellular carcinoma (HCC) has a decisive influence on the prognosis of cirrhotic patients. Although α -fetoprotein (AFP) is a known and specific tumor marker for HCC, it is not suitable for the screening and surveillance of HCC because of its poor predictive value and low sensitivity. The use of imaging modalities is essential for the screening, diagnosis and treatment of HCC. Ultrasound (US) plays a major role among them, because it provides real-time and non-invasive observation by a simple and easy technique. In addition, US-guided needle puncture methods are frequently required for the diagnosis and/or treatment process of HCC. The development of digital technology has led to the detection of blood flow by color Doppler US, and the sensitivity for detecting tumor vascularity has shown remarkable improvement with the introduction of microbubble contrast agents. Moreover, near real-time 3-dimensional US images are now available. As for the treatment of HCC, high intensity focused ultrasound (HIFU) was developed as a novel technology that provides a transcutaneous ablation effect without needle puncture. These advancements in the US field have led to rapid progress in HCC management, and continuing advances are expected. This article reviews the current application of US for HCC in clinical practice.

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Key words: Ultrasound; Contrast agent; Hepatocellular carcinoma; Liver; Surveillance; Treatments

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INTRODUCTION

Hepatocellular carcinoma (HCC) is increasing worldwide and is one of the most common carcinomas in the eastern part of Asia^[1]. As the prognosis of cirrhotic patients depends on the occurrence and progression of HCC, management of this neoplasm is a major issue in clinical practice. The recent popularization of periodic surveillance and the development of diagnostic capabilities have resulted in the discovery of increasing numbers of patients with small HCC nodules^[2,3]. Although tumor markers may be helpful for the diagnosis of HCC, imaging modalities are essential for finding and characterizing this neoplasm^[4,5].

On the basis of the continuing development of digital technologies, ultrasound (US) has also shown significant improvements within the last decade^[6]. As for grey-scale imaging, tissue harmonic imaging (THI) has improved both lateral resolution and contrast resolution by narrowing the width of the US beam, with the reduction of reverberation and side-lobe artifacts. Since the margin and structure of tumor nodules have become clear, with distinct delineation^[7-10], THI has become popular as part of the routine work of grey-scale US examination.

Color Doppler imaging provides real-time evaluation of the hemodynamics in liver tumors, and power Doppler mode has contributed to a better detectability of blood flow^[11-15]. However, limitations in the detection of slow flow and vessels located deeply from the skin surface have prevented the wider application of Doppler mode in the evaluation of tumor hemodynamics^[16-18]. Furthermore, artifacts caused by respiratory or cardiac motion sometimes affect the precise evaluation of hemodynamic information.

With these backgrounds, US contrast agents have been expected to improve the detectability of blood flow in liver tumors, since the first report about a US contrast agent by Gramiak *et al*^[19]. From the late 1980s to the 1990s, grey-scale contrast-enhanced US with carbon dioxide gained broad attention as an echo-enhancing technique, with

high sensitivity for detecting tumor vascularity and high performance for the characterization of liver tumors^[20,21]. However, this method requires an arteriography procedure because carbon dioxide is easily soluble in blood. The development of microbubble contrast agents together with peripheral venous injection was expected for practical use. In the late 1990's, a galactose-based US contrast agent (SHU 508, Levovist) was made available by Schering, Germany^[22,23]. It was a long-awaited material that could provide a stable enhancement effect in abdominal organs with a peripheral injection. Subsequently, many microbubble contrast agents have been produced or are currently under development. At present, the application of Doppler mode alone for detecting tumor blood flow is rare, as contrast-enhanced US with microbubble contrast agents provides details of the hemodynamics that are useful for the detection and characterization of liver tumors. Additionally, three-dimensional US images are now easily available due to the development of advanced digital technologies^[24,25], and high intensity focused ultrasound (HIFU) was developed as a novel treatment method for tumors^[26]. This article reviews the current development and application of US for the diagnosis and treatment of HCC.

SURVEILLANCE FOR HCC

Viral-related and/or alcoholic chronic liver disease is a high-risk factor for developing HCC that limits the prognosis. There is no question about the importance of periodic surveillance for HCC in these high-risk patients^[27-29]. Some serum markers are known for HCC, and α -fetoprotein (AFP) is widely used for its diagnosis^[30-32]. Ishii *et al* reported that sensitivity and specificity of AFP was 13.8% and 97.4% at a cut-off value of 200 ng/mL, respectively, and 62.1% and 78.3%, at a cut-off value of 20 ng/mL, respectively^[31]. They added that when AFP and another tumor marker, protein induced by vitamin K absence or antagonist II (PIVKA-II), were combined with cut-off values of 40 ng/mL for AFP and 80 mAU/mL for PIVKA-II, sensitivity was 65.5% and specificity was 85.5%. The study by Tong *et al* showed that the positive predictive value for AFP to detect HCC was only 12% or less for all AFP cut-off values, and the maximum joint sensitivity and specificity as determined by receiver operator characteristic (ROC) analysis were approximately 65% and 90%, respectively. Meanwhile, the positive predictive value for US to detect HCC was 78%, while sensitivity and specificity were 100% and 98%, respectively^[33]. They concluded that AFP should not be used as the only test for screening and surveillance for HCC because of its poor predictive value and low sensitivity. Larcos *et al* also mentioned that US screening was superior to AFP assay for detection of HCC^[34]. Novel serum markers with improved sensitivity are awaited for screening tests for HCC.

US is the most common method for the screening of HCC because of its advantages - simple, non-invasive and real-time observation^[4,5]. However, there has been a variety of results in the application of US for HCC surveillance (Table 1). Sherman *et al* reported that US

Table 1 Sensitivity and specificity of US and other imaging modalities for the screening of HCC

Authors	US		Other modalities	
	Sensitivity (%)	Specificity (%)	Sensitivity (%)	Specificity (%)
Sherman <i>et al</i> ^[35]	71.4	93.8	-	-
Chalasani <i>et al</i> ^[36]	59	93	91 (CT)	96 (CT)
¹ Yao <i>et al</i> ^[37]	79.4	-	81.6 (CT)	-
			88.9 (MRI)	-
Gambarin-Gelwan <i>et al</i> ^[38]	58	94	53 (CT)	94 (CT)
² Teefey <i>et al</i> ^[39]	89	75	67 (CT)	75 (CT)
			56 (MRI)	81 (MRI)
			0 (PET)	88 (PET)

¹Sensitivity of radiologic procedures in the diagnosis and staging of known HCC before liver transplantation. ²The higher value was presented from two data obtained between two observers.

showed a sensitivity of 71.4%, a specificity of 93.8%, with only 14% of positive predictive value, as a screening test in chronic HBsAg carriers^[35]. Chalasani *et al* compared the sensitivity in a screening program between US and computed tomography (CT), and the sensitivity of US (59%) was much lower than that of CT (91%)^[36]. Two other studies in the diagnosis of HCC before liver transplantation resulted in similar sensitivity between US and CT, 79.4% for US and 81.6% for CT^[37], 58% for US and 53% for CT^[38], respectively, with the latter claiming that US is preferable to CT for routine screening of HCC before liver transplantation because of its lower cost. Meanwhile, Teefey *et al* mentioned that the sensitivity of US (89%) was much higher than CT (67%) and magnetic resonance imaging (MRI, 56%)^[39]. Evaluation of the actual sensitivity of US and other imaging techniques from the published studies on screening and surveillance is quite difficult because of the lack of a defined gold standard, as was also noted in the review article by Bolondi^[28]. In addition, Chalasani *et al* described in their study that the lesser steatosis to change liver echogenicity in Asian patients with predominantly viral cirrhosis, leaner body habitus in Japanese patients resulting in better visualization of the liver by US, and differences in US technique between physicians (Japan) and technologists (USA) were the causes for the high detection rates by US in Japanese reports^[4,36,40]. Although it is natural that US results depend on the physical size of the patients and the operator's skill, medical staffs and engineers who engage in US should not accept the current situation. Further technical and technological improvements are required to overcome these problems.

Tumor detectability between US without enhancement and contrast-enhanced spiral CT has been compared in some previous studies. The comparison may not be on an equal footing, as US has now acquired collaboration with microbubble contrast agents. The application of contrast-enhanced CT for screening of HCC would be expensive and invasive, and MRI has the limitation of a low availability rate of the equipment. Although contrast-enhanced US may not be cheap, it is much less invasive and more convenient than contrast-enhanced CT. The

establishment of surveillance based on both non-contrast US and contrast-enhanced US may be necessary for the screening procedure of HCC.

According to clinical studies concerning the doubling time of tumor, median days were reported as 117 d (29-398 d) by Sheu *et al*^[27] or 171.6 d (27.2-605.6 d) by Barbara *et al*^[41], and the former study called for a suitable screening interval for the early detection of HCC of 4-5 mo. Solmi *et al* reported that the percentage of detected unifocal tumors with a diameter less than or equal to 3 cm was significantly higher in the group followed-up every six months by both US and AFP than the group without this follow-up protocol^[42]. Depending on the risk factors, a score based on certain clinical findings may be predictive for the doubling time of HCC^[41,43]. The latter report recommended a regular US follow-up of a 3- or 6-mo interval according to the risk of HCC development, sex (male), alkaline phosphatase, AFP, γ -glutamyltransferase and albumin^[43]. The study by Izzo *et al* also supported the 6-month surveillance by AFP and US for patients with severe chronic active hepatitis or liver cirrhosis^[44]. However, Fasani *et al* reported that screening with US every six months may be inadequate for early detection of liver cancer in patients with multiple risk factors because multinodular HCC was under detected by US^[45]. A tailor-made surveillance interval may be required according to the risk of HCC development.

Bolondi *et al* examined their surveillance program based on US and AFP at six-month intervals in 313 cirrhotic patients, reporting that the cumulative survival of the 61 patients with liver tumors detected by the surveillance program was significantly longer than that of controls not participating in any specific surveillance program, with incidentally detected HCC, and multivariate analysis showed an association between surveillance and survival^[46]. Other studies showed that surveillance based on US and AFP every 6-12 mo improved the survival of patients^[47,48].

As described above, the method and appropriate interval of surveillance have been discussed from the aspect of growth speed of HCC, detected number and size of HCC, and the risk of developing HCC. Furthermore, the significance of surveillance is well-supported by the improved survival rate. US should play a main role in the screening procedure of HCC.

DIAGNOSIS OF HCC

Imaging diagnosis of HCC is based on the presentation of characteristic hypervascular appearances in nodules. The European Association for the Study of the Liver (EASL) has documented the diagnostic criteria for HCC in a report for the clinical management of HCC^[49]. Nodules larger than 2 cm with an arterial hypervascular pattern by two imaging techniques or by one imaging technique associated with an AFP level higher than 400 ng/mL was considered to be HCC in cirrhotic patients without needing confirmation by a positive biopsy. Four imaging modalities, US, spiral CT, MRI, and angiography, were recommended for evaluation of the vascularity of hepatic nodules in that article.

The advantages of US imaging consist of the simple

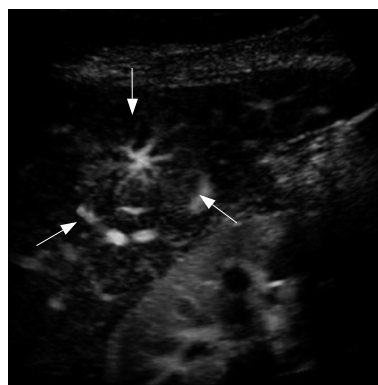


Figure 1 Contrast-enhanced harmonic imaging with Sonazoid in focal nodular hyperplasia (FNH). The centrifugal blood flow appearance like "spoke-wheel sign" was clearly demonstrated in the center of the nodules (arrows).

and non-invasive demonstration of blood flow by real-time observation. US is a unique method that can evaluate blood flow direction under physiological condition. In contrast to focal nodular hyperplasia (FNH) with a centrifugal blood flow appearance (Figure 1), HCC has a characteristic hypervascular appearance with centripetal blood flow, and a basket pattern is one of the typical findings of HCC by color Doppler imaging^[50-52]. The clinical application of microbubble contrast agents has resulted in remarkable improvement in blood flow detection by US examination. It was reported that the same enhancement pattern was found between contrast-enhanced harmonic grey-scale imaging with Levovist and contrast-enhanced helical CT in 53 of 61 (87%) HCC nodules^[53]. Other studies have also shown over 80% concordance of tumor vascularity^[54,55] between contrast-enhanced US with SonoVue (Bracco Diagnostics, Princeton, NJ, USA) and contrast-enhanced helical CT. Thus, the application of Doppler mode alone for detecting tumor blood flow is rare, as the more recent availability of microbubble contrast agents has assisted in overcoming the limitations of Doppler methods.

The diagnostic performance of contrast-enhanced US is not limited to the demonstration of tumor vascularity. Some microbubble contrast agents have a characteristic property of organ-specific accumulation^[56-59]. Although the precise mechanism remains unclear, the reticuloendothelial system (i.e., phagocytosis by Kupffer cells) may be involved in this phenomenon. Both Levovist and Sonazoid (Nycomed-Amersham, Oslo, Norway) accumulate in the liver, and sonograms in this phase (late liver-specific parenchymal phase) are frequently used for the detection or characterization of liver tumors. In contrast, Definity (Bristol-Myers Squibb, N. Billerica, MA, USA) and SonoVue do not accumulate in the liver. The characterization of liver tumors by contrast-enhanced US has been carried out using accumulation images as well as vascular enhancement images (Figure 2A and B).

Concerning the discrimination of malignant versus benign liver lesions by contrast-enhanced US, recent literature has reported sensitivity of 98% to 100% and specificity of 63% to 93% with Levovist^[60-63], and sensitivity of 98% and accuracy of 92.7% with SonoVue^[64]. Furthermore, in a clinical study with two independent image reviewers, Kim *et al*^[65] described that contrast-enhanced US (agent detecting imaging mode with Levovist) provided a specific diagnosis in 75%-79% of 75 patients with focal hepatic lesions, and that the technique

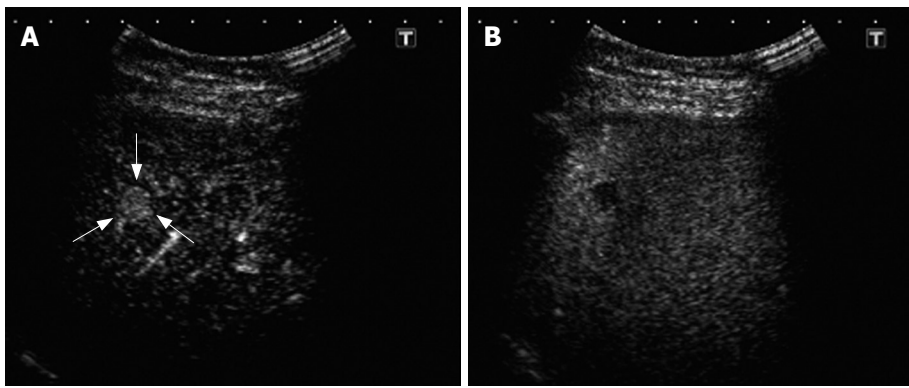


Figure 2 Contrast-enhanced harmonic imaging with Sonazoid in small HCC (9.8 mm, arrows). **A:** Early-phase image (22 s after the injection); **B:** Late-phase image (10 min after the injection). The early-phase image showed positive enhancement and the late-phase image showed negative enhancement in the nodule. These findings could easily diagnose this lesion as HCC.

was successful as a confirmatory imaging technique in 63%-72% of the patients.

Hypervascular hepatic lesions do not always reflect the fact that the final diagnosis of the nodule is HCC in heavy drinkers^[66], since benign hypervascular nodules sometimes occur in their liver. A recent report has shown that the ring-shaped appearance on liver-specific contrast-enhanced sonograms with Levovist may be a useful sign for the differential diagnosis of benign nodule from HCC in heavy drinkers^[67]. Since contrast-enhanced CT hardly differentiates these benign nodules from HCC, this characteristic finding may prevent unnecessary treatments under misdiagnosis. Moreover, it could be expected to lead to a reduction in the application of percutaneous needle biopsy, an invasive procedure, for the precise diagnosis.

Non-hypervascular and/or small (< 2 cm) nodules

Well-differentiated HCC, dysplastic nodule (DN) and regenerative nodule (RN) do not always reveal the specific hypervascular pattern on contrast-enhanced CT such as typical HCC^[68-71]. The characterization of such non-hypervascular nodules is very important in clinical practice^[72,73] because high-grade DN are considered potentially pre-malignant lesions. However, as these non-hypervascular nodules have Kupffer cell distribution^[74,75], observation of the superparamagnetic iron oxide-enhanced (SPIO) MR images or liver-specific images on contrast-enhanced US could not easily characterize them.

According to the EASL report, percutaneous needle biopsy has until now been a standard method for the diagnosis of non-hypervascular hepatic nodules or small hepatic nodules of 1 cm to 2 cm^[49], because characterization of these nodules by imaging modalities alone is difficult^[76-79]. As for nodules smaller than 1 cm, EASL recommended repeated US observation every 3 mo until the lesion grows to 1 cm, at which point additional diagnostic techniques can be applied^[49].

Thanks to the establishment of US-guided needle puncture technique^[80], percutaneous needle biopsy has a quite high diagnostic accuracy. Caturelli *et al* found that the typing accuracy of fine-needle aspiration biopsy was 88.6% for nodules with diameters < 10 mm, 86.2% for nodules with diameters of 11-15 mm, and 91.3% for nodules with diameters of 16-20 mm^[81]. Durand *et al* reported that US-guided FNB diagnosed HCC nodules with a sensitivity of 91%^[82]. However, liver biopsy for small nodules always has the possibility of sampling error, and a negative biopsy of

a nodule visible with imaging techniques in a cirrhotic liver can never be taken as a criterion to rule out malignancy^[83]. Additionally, as rapid progression is rare in these kinds of nodules, repeated observations in their clinical course would determine their management. Therefore contrast-enhanced US can be expected to be an effective diagnostic tool for these non-hypervascular lesions because of its high resolution and non-invasive procedure.

TREATMENT SUPPORT AND EVALUATION OF THERAPEUTIC EFFECT

US-guided treatment

Since the majority of HCC patients have poor liver function and recurrence is not rare, surgical treatment is not always an appropriate choice^[2,3,49]. With such backgrounds, percutaneous ethanol injection (PEI)^[84-86] and radio-frequency ablation (RFA)^[87,88] were developed and came to be widely used in clinical practice as minimally invasive methods^[89]. They are now a first-line, favored approach with an efficient therapeutic effect on HCC^[90-93].

Treatment for recurrent lesions

Although percutaneous US-guided treatments provide sufficient therapeutic effect, recurrence often plagues many HCC patients. According to long-term study results, cumulative recurrence rates of the treated site of post-PEI lesions were 3.4% at 1 year, 7.1% at 2 years, and 10% at 3 years, and those of the untreated sites in liver were 18.7% at 1 year, 62.1% at 3 years, and 81.7% at 5 years, respectively^[94]. Thus, many HCC patients have to receive repeated treatments during their clinical course. In order to minimize adverse effects to the liver, less invasive treatment such as PEI or RFA is preferable for these patients. However, localization of lesions on sonograms is sometimes problematic in patients with cirrhotic liver and/or repeated treatment history^[95,96]. Although percutaneous treatment under CT guidance is a well-established technique and a useful method for lesions undetected by US, the method lacks convenience and exposes both patients and physicians to radiation^[97-100]. Microbubble contrast agents are also useful in such a case. A recent study showed that contrast-enhanced US with Levovist could localize 24/32 (75%) of HCC lesions that were invisible by non-contrast US^[101]. Application of the next-

generation US contrast agents, SonoVue and Sonazoid, is expected to improve the localization result.

Evaluation of therapeutic effect

US examination is eligible for the evaluation of the therapeutic effect after percutaneous treatments such as PEI and RFA, because they are usually performed under US guidance. In fact, contrast-enhanced US has come to be frequently applied for evaluation of the therapeutic response in HCC nodules with improved sensitivity and specificity for detecting tumor blood flow (Table 2). According to the results by Bartolozzi *et al*, color Doppler US with Levovist showed sensitivity of 92%, specificity of 100%, and accuracy of 98% compared to the results of spiral CT and biopsy, in the detection of residual tumor tissue in 47 HCC lesions after PEI^[102]. Wen *et al* examined the efficacy of coded harmonic angio mode with Levovist for detecting residual tumor in 91 HCC nodules about one week after RFA in comparison with contrast-enhanced CT, and they found that sensitivity, specificity, and diagnostic accuracy of US were 95.3%, 100%, and 98.1%, respectively^[103]. Meloni *et al* reported that sensitivity and specificity of pulse inversion harmonic imaging with Levovist were 83.3% and 100%, respectively, for detecting residual non-ablated tumor at 4 mo after treatment in 35 patients with 43 HCC nodules, compared with helical CT findings^[104]. Immediate evaluation of the therapeutic effect is often desirable after RFA for the management of HCC, and Choi *et al* mentioned that diagnostic agreement between power Doppler with Levovist about half or one day after ablation therapy and CT just after ablation was achieved in 100% of the 45 HCC nodules in 40 patients^[105]. Another study showed that diagnostic concordance between agent detection imaging with Levovist performed within 24 h after RFA and 1-mo follow-up CT was 99% in 90 patients with 97 HCC nodules^[106]. Thus, estimation of the therapeutic response in HCC after percutaneous treatments would become more efficient on the basis of this non-invasive imaging method. Although artificial signals caused by the RFA procedure affect an early detailed observation^[105-107], monitoring by contrast-enhanced US during RFA would likely be applied to the assessment of the therapeutic effect as well as the detection of viable tumor.

It is well known that contrast-enhanced CT can hardly evaluate intratumoral contrast enhancement when partial retention of iodized oil is present in the tumor after transcatheter arterial chemoembolization (TACE). Therefore, the therapeutic effect of TACE is usually assessed by the distribution of iodized oil in the tumor on non-contrast CT images, though these findings are an indirect presentation. As MRI findings are not affected by the presence of iodized oil, contrast-enhanced MRI is favorable for the assessment of the therapeutic effect after TACE. However, the equipment has not yet come into wide-spread use, the procedure is not convenient, and evaluation of the findings in small lesions is sometimes difficult due to the low resolution and influence of motion artifacts. Contrast-enhanced US has the advantage of not being limited by iodized oil deposition that affects

Table 2 Assessment of therapeutic response after percutaneous treatment for HCC using contrast-enhanced US

Author	Treatment	No. of patients/ No. of lesions	Results ¹ (contrast agent)
Bartolozzi <i>et al</i> ^[102]	PEI	40/47	Sensitivity 92% Specificity 100% Accuracy 98% (Levovist)
Wen <i>et al</i> ^[103]	RFA	67/91	Sensitivity 95.30% Specificity 100% Accuracy 98.10% (Levovist)
Meloni <i>et al</i> ^[104]	RFA	25/43	Sensitivity 83.30% Specificity 100% (Levovist)
Choi <i>et al</i> ^[105]	RFA	40/45	Diagnostic agreement 100% (Levovist)
Kim <i>et al</i> ^[106]	RFA	90/94	Diagnostic concordance ² 99% (Levovist)
Solbiati <i>et al</i> ^[107]	RFA	20/20 ³	Sensitivity 50% Specificity 100% Diagnostic agreement 85% (Levovist)
Pompili <i>et al</i> ^[110]	PEI, RFA, TACE Combined treatments	47/56	Sensitivity 87% Specificity 98.40% Diagnostic agreement 94.60% (SonoVue)

¹Comparison with contrast-enhanced helical CT; ²1-mo follow-up CT;

³Solitary colorectal liver metastases.

the evaluation of contrast-enhanced CT findings. Some clinical studies have shown the magnitude of contrast-enhanced US for evaluation of the therapeutic effect after TACE^[108,109]. According to the report by Pompili *et al*, contrast-enhanced US with SonoVue resulted in diagnostic agreement in 53/56 cases (94.6%), with 87.0% sensitivity and 98.4% specificity compared with contrast-enhanced CT findings, after non-surgical treatments for HCC^[110]. Another study showed that contrast-enhanced US resulted in considerably higher sensitivity in detecting residual tumor blood flow after TACE than dynamic CT or dynamic MRI^[111]. Meanwhile, Lim *et al* described that a reliable assessment of intratumoral blood flow by contrast-enhanced US may not be possible in many instances, particularly in small lesions or in lesions located deep within the liver parenchyma^[112]. They concluded that CT is the standard imaging technique for monitoring the effectiveness of TACE and RFA, and contrast-enhanced US and MRI can complement CT in evaluating the therapeutic response. Although the performance of the US examination may depend on the operator's skill, location of the tumor and system capability, quite a few radiologists and hepatologists may believe that contrast-enhanced US plays a major role in evaluation of the therapeutic effect after TACE. The recent developments in this technology would allow contrast-enhanced US to be positioned as the standard method for evaluation of the therapeutic effect in many HCC patients (Figure 3A and B).

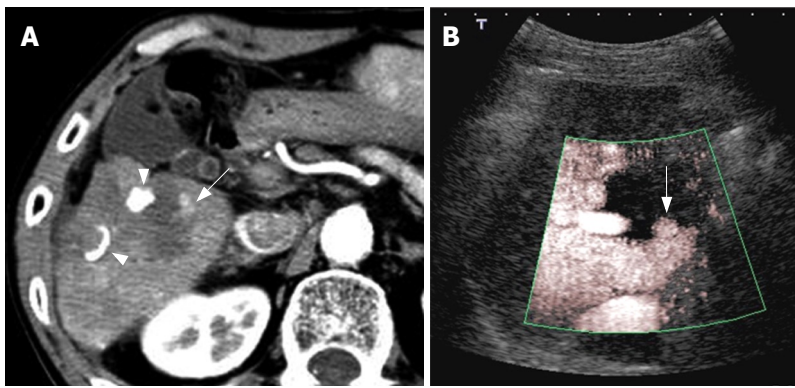


Figure 3 Assessment of therapeutic response after PEI for HCC. **A:** Contrast-enhanced CT with dynamic study; **B:** Contrast-enhanced US (Advanced Dynamic Flow with Levovist). Contrast-enhanced CT showed enhancement appearance which needed additional treatment within the treated area (arrow), and contrast-enhanced US could demonstrate a similar finding. Arrow heads: Lipiodol.

ADVANCED TECHNOLOGY

Recent US systems have provided three-dimensional visualization of the combined tissue structures and color blood-flow appearance under easy handling^[24,25]. Additional anatomical information of the tumor with tumor-associated vessels is available at any plane from multiple directions^[113-116]. With the remarkable progress in microelectronic technology, the US transducer has achieved full digital specification (Matrix transducer, iu22, Philips) with 3000 elements^[117,118]. Including built-in micro-beamforming composed of a 150-computer board, it can visualize “Live 3D”, which presents real-time three-dimensional anatomical views visible from any angle with volume rendering for pyramidal volume (90°×70° angles). Contrast-enhanced 3D or 4D ultrasonographies using microbubble contrast agents might become a standard method for the characterization and/or evaluation of the therapeutic effect on liver tumors (Figure 4)^[119].

HIFU is a novel technology that enables transcutaneous ablation effect without needle puncture^[120,121]. While controlling the energy and focusing of US, successful HIFU results in necrosis of the tumor in the focal area with less damage of surrounding tissues. A number of clinical studies have been carried out using HIFU for the treatment of liver tumors as well as breast cancer and myoma uteri. In regard to liver tumors, it was reported that the anti-tumor effect and survival time by HIFU combined with TACE were superior to those by TACE alone in 50 patients with advanced HCC^[122]. Although some of the subjects seemed to have a complete ablation effect, the precise effect for complete tumor necrosis by HIFU was not clear in this study. Furthermore, as the background of the HCCs showing sufficient ablation effect was not fully analyzed, it remains to be solved whether HIFU is valuable as a reliable method for curative treatment of small HCC. Nonetheless, this non-invasive method is really expected to be used for HCC treatment, as an alternative to PEI or RFA, because needle puncture is an invasive procedure for cirrhotic patients.

Normal ventilation is one of the serious problems in the completion of HIFU treatment for liver tumor, as movement of the liver may cause ablation failure that results on non-tumor tissue damage and/or incomplete therapeutic effect for the tumor. Wu *et al.* reported that three-dimensional US images were used as a monitor to localize the tumor during HIFU treatment, and changes

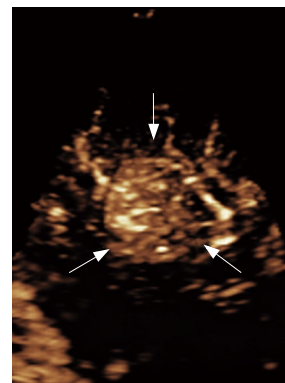


Figure 4 Real-time three-dimensional imaging of HCC (contrast-enhanced LIVE 3D with Sonazoid, iu22, Philips). Abundant tumor vessels were dramatically demonstrated in the HCC nodule. (Arrows: HCC nodule).

in echogenicity of the tumor just after the treatment were evaluated by US^[122]. Advances in imaging technology for real-time 3D sonography would help the improvement of the therapeutic ability of HIFU.

In conclusion, US has made amazing strides in the last decades because of digital technology progress, and it will continue to grow. The advancement of imaging methods is expected to support the clinical management of patients with HCC.

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TOPIC HIGHLIGHT

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Tumor suppressor and hepatocellular carcinoma

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Abstract

A few signaling pathways are driving the growth of hepatocellular carcinoma. Each of these pathways possesses negative regulators. These enzymes, which normally suppress unchecked cell proliferation, are circumvented in the oncogenic process, either the over-activity of oncogenes is sufficient to annihilate the activity of tumor suppressors or tumor suppressors have been rendered ineffective. The loss of several key tumor suppressors has been described in hepatocellular carcinoma. Here, we systematically review the evidence implicating tumor suppressors in the development of hepatocellular carcinoma.

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Key words: Tumor suppressor; Hepatocellular carcinoma; Deregulation; Liver; Carcinogenesis

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the most common form of primary hepatic tumor and is one of the most common cancers worldwide. HCC usually develops in patients with cirrhosis. Cirrhosis may be caused by viral hepatitis (primarily hepatitis B and C), alcohol, hereditary

haemochromatosis, autoimmune liver diseases and actually any disease that results in chronic inflammation of the liver. In order to understand how chronic inflammation and cirrhosis lead to initiation and progression of HCC extensive research on molecular events has been undertaken. Several intracellular signaling pathways have been closely associated with HCC: p53 pathway and DNA alteration, retinoblastoma (Rb) pathway and regulation of cell cycle, transforming growth factor-beta (TGF- β) and inhibition of cellular growth, and Wnt/beta-catenin pathway and cellular adhesion and signal transduction^[1]. Deregulation in these different pathways favors the development of liver tumor. Conceptually, hepatocarcinogenesis is based on two main principles: (1) the activation of genes such as oncogenes (c-myc, β -catenin), growth factor (IGF-II, TGF- α) and telomerase enzyme inducing cellular immortalization and (2) the inactivation of genes called tumor suppressor genes (for example p53 and Rb) Their expressions can be affected by different modifications such as promoter methylation, mutations, biallelic loss and loss of heterozygosity (LOH). In liver cancer, chromosomal aberrations are frequently observed on chromosome 1, 4, 5, 6, 8, 9, 10, 11, 13, 16, 17, 20 and 22 sharing the complexity of hepatocarcinogenesis. Moreover, if some mutations are associated with initiation of carcinogenesis, other genetic alterations promote progression and clone divergence in tumors^[2,3].

This review addresses the various tumor suppressors, which have been implicated in HCC (Table 1). Specifically, we discuss their function and involvement in the initiation or progression of HCC as well as their mechanisms of inactivation.

p53 AND ITS PATHWAY

p53

TP 53 gene, located on chromosome 17p13.1, encodes a 53 kDa DNA-binding transcription factor. The protein p53 is implicated in the control of cell cycle, apoptosis, DNA repair and angiogenesis. p53 activation has been related to various cellular and environmental changes including: (1) DNA damage (induced by UV light, gamma rays, X rays, and inhibition of topoisomerases), (2) cellular stress (hypoxia, disruption of cell adhesion) independent of DNA damage and (3) activation of growth signaling pathways^[4,5]. p53 gene is a haploinsufficient tumor suppressor gene^[6,7]. The loss of p53 activity has been described in many types of human tumors, particularly

Table 1 Summary of tumor suppressor regulated in HCC

Symbol	Name	Location	Haplo-insufficiency	Downregulation in HCC	Mechanism of regulation				Etiology
					Mutation	Methylation	Chromosome change	Protein overcrossing	
p53 ^[3,12-14,21,23,29,31-33,180]	Tumor protein p53	17p13.1	Yes	Yes	✓	✓			AFB1 HBV HCV Cirrhosis
p21 ^[51-53]	Cyclin-dependent kinase inhibitor 1A	6p21.2	Yes	Yes				p53	HBV HCV cirrhosis
p27 ^[60]	Cyclin-dependent kinase inhibitor 1B	12p13.1-12p	Yes	Yes		✓			
p16 ^[38, 66-68]	Cyclin-dependent kinase inhibitor 2A	9p21		Yes	✓	✓			AFB1
p14ARF ^[38]	Cyclin-dependent kinase inhibitor 2A	9p21		Yes	✓	✓			HCV Cirrhosis
E-cadherin ^[70-73]	E-cadherin	16q22.1		Yes		✓			HBV HCV
Axin 1 ^[74-76,78,79]	Axis inhibitor 1	16p13.3		Yes	✓		✓		HBV HCV
Axin 2 ^[75,78,79]	Axis inhibitor 2	17q23-q24		Yes	✓		✓		
APC ^[81-84]	Adenomatosis polyposis coli	5q21-q22		Yes	✓	✓	✓		HBV HCV
SOCS1 ^[89-90]	Suppressor of cytokine signaling 1	16p13.13		Yes		✓	✓		HCV Cirrhosis
SOCS3 ^[93,94]	Suppressor of cytokine signaling 3	17q25.3		Yes		✓			
RASSF1A ^[83,104,105]	Ras association (ralGDS/AF-6) domain family 1	3p21.3		Yes		✓	✓		AFB1 HBV HCV
NORE1 ^[94-108]	Ras association (ralGDS/AF-6) domain family 5	1q32.1		Yes		✓			HBV HCV Cirrhosis
KLF6 ^[116,119]	Kruppel-like factor 6	10p15		Yes	✓	✓	✓		HBV HCV Cirrhosis
PTEN ^[123,127,128,130]	Phosphatase and tensin homolog	10q23.3	Yes	Yes	✓			mTOR	AFB1 HBV
Hint1	Histidine triad nucleotide binding protein	5q31.2	Yes	n.d	n.d	n.d	n.d	n.d	
Hint2 ^[144]	Histidine triad nucleotide binding protein 2	9p13.3		Yes	n.d	n.d	n.d	n.d	
FHIT ^[149-151,154,157]	Fragile histidine triad gene	3p14.2	Yes	Yes		✓	✓		HBV HCV Cirrhosis
WWOX ^[160]	WW domain containing oxidoreductase	16q23.3-q24		Yes			✓		AFB1
PARK2 ^[173]	Parkin	6q25.2-q27		Yes					

in 30%-60% of HCC. In many cases, these alterations contribute to progression and not to initiation of HCC^[8]. In terms of prognosis, p53 alterations are generally associated with larger, less differentiated tumors and poor survival^[9]. Recently, Lowe and its team observed that senescence program in correlation with the innate immune system turn off the tumor development after restoration of p53 expression in liver tumor cells^[10]. Different studies described that p53 is regulated by methylation of its CpG islands in HCC^[11,12] but its expression is mainly regulated by genetic mutations. Mutations affecting p53 are diverse by their nature and position. The p53 gene is altered by allelic deletion and punctual mutation concentrated between exons 4 and 9 of the coding region containing the DNA binding domain. Amongst these mutations, the transversion in codon 249 (G→T), which causes an

arginine to serine (R→S) substitution is present in 50% of HCCs. This genetic alteration can be a consequence of exposure to aflatoxin B1 (AFB1) which is a mycotoxin found in contaminated foods (like corn, rice, and peanuts) particularly in African and Asian countries^[13,14]. Kirk have proposed the use of p53 mutant DNA as a biomarker for AFB1 exposure^[15]. Mutated R249S p53 protein expression may induce (1) an inhibition of apoptosis^[16], (2) inhibition of p53 mediated transcription^[17] and (3) stimulation of liver cell growth *in vitro*^[18-20]. Like AFB1, the hepatitis B virus (HBV) affects the activity of p53 by inducing DNA damage and mutating the p53 gene. Synergism between AFB1 and HBV has been described^[21,22]. The X gene of HBV (HBx) encodes a protein of 154 amino acids, which is a viral transcriptional co-activator capable of activating the expression of several proteins such as oncogenes

(c-myc, c-fos), cellular growth factors and cytokines. Protein X inactivates various functions of p53^[3,23] such as apoptosis^[17,24-27] and transcriptional activation^[28]. Concerning HCV, the non-structural protein NS2-5 seems to deregulate the actions of factors controlling hepatocellular proliferation by inhibiting p21^{WAF} and sequestering p53^[29]. In transgenic mice, HCV core protein has been found *in vivo* to stimulate the initiation and development of tumor with the same histological characteristics of human HCC^[30]. HCV core protein may interact directly with p53 and p73^[31-33].

In HCC, p53 level and activity are modulated by different proteins such as MDM2 (murine double minute 2) and p14^{ARF} (Alternative Reading Frame). Oncogenic MDM2 protein contains a p53-DNA binding site and induces p53 degradation by ubiquitination and proteolysis^[34,35]. However, auto-regulatory feedback loop of MDM2-p53 controls the function and expression of p53 and MDM2, respectively^[36]. However, the activity of MDM2 is inhibited by ARF tumor suppressor protein^[37]. This protein is encoded by INK4a/ARF locus on chromosome 9p21, which is frequently affected by hypermethylation and mutations in HCC^[38]. In the clinical treatment strategy of HCC, the reactivation of p53 protein is focused on stabilization of p53 activity, over-expression of ARF protein and inactivation of MDM2-p53 interaction^[39]. In animals, different small molecules such as nutlins and PRIMA-1 inactivate MDM2 and increase p53 activity^[40,41]. Loss of cell cycle check-point control by mutation of p53 has been suggested to stimulate the metastatic potential of HCC via over-expression of L2DTL^[42]. Ubiquitination and protein stability of p53 are regulated by a complex regrouping L2DLT, CDT2 and PCNA^[43]. In contrast, TIP30, which is known to inhibit cell proliferation and tumorigenesis, may act as a hepatocarcinogenic tumour suppressor^[44]. This protein diminishes Bcl2/Bcl-x expression and augments p53 expression^[45]. TIP30 mutants inhibited the expression of tumor suppressor genes such as p53 and E-cadherin whereas they induced positive regulation of oncogenic genes expression such as N-cadherin and c-Myc^[46]. Immunohistochemical analysis of HCC and normal liver showed that a 33 kDa protein called ING1 (inhibitor of growth-1, p33 (ING1)) is expressed at a lower level in HCC. Lower ING1 protein level was associated with enhanced cyclin E kinase activity^[47]. Recently, Zhu and co-workers proposed that p33 (ING1b) and p53 work in tandem to enhance apoptosis, cell cycle arrest and to inhibit cell growth in HCC. Combined expression of p33 and p53 augments p21 (WAF1/CIP1) protein causing an arrest of cell cycle at stage G0/G1 and enhancing apoptosis^[48]. The p53 pathway is intercrossing with other tumor suppressors as p21, p27 and p16, which are described below.

p21

p53 directly controls a gene encoding for a protein named p21WAF/CIP1 (p21). This protein, whose gene is located on chromosome 6p21.2, acts as haploinsufficient tumor suppressor gene^[49]. It functions as an inhibitor of cyclin-dependent kinases (cyclin-CDK2, CDK4) and interacts with proliferating cell nuclear antigen

(PCNA), a DNA polymerase accessory factor. This protein has a regulatory role in the cell cycle, S phase DNA replication and DNA damage repair. p21 has also been described to activate the caspase 3 protein and thus induce apoptosis. A reduction of p21 expression was observed in HCC^[50]. Moreover, tumor progression and poor outcome of HCC were associated at disruption of p53-p21/WAF1 cell cycle pathways^[51]. In a study addressing p53 expression and apoptosis, high p53 expression was associated with cell cycle arrest and apoptosis whilst a lower level of p53 induced only cell cycle arrest. However, p21 expression could activate only cycle arrest but not apoptosis in HCC as well as in the presence of high p53 as low p53 expression^[52]. The transcription of p21 gene was found to be repressed by HBV X protein (HBx) and HCV core protein^[53]. In addition, the stress due to the inflammation and fibrosis of HCV-associated chronic liver diseases induced up-regulation of p21^[54]. Huether and co-workers analyzed the effect of cetuximab (Erbix), a chimeric human/mouse antibody directed against the Epidermal Growth Factor Receptor (EGFR), with or not in combination with other drugs on human hepatocellular carcinoma cell lines. The expression of the cyclin-dependent kinase inhibitors p21 and p27 (Kip1) was increased whereas the expression of cyclin D1 was decreased by cetuximab treatment. A synergistic antiproliferative effect was observed following a treatment with cetuximab combined with doxorubicin, tyrosine kinase inhibitors (erlotinib or AG1024) or the HMG-CoA-reductase inhibitor fluvastatin^[55]. The expression of different proteins such as p53, p21 cyclin D implicated in enhancement of the apoptosis pathway and cell proliferation have been analyzed after treatment with antiangiogenic agent TNP-470 in a rat model of hepatocellular carcinoma. The augmentation in these angiogenic factors induced by HCC was prevented whereas a cell-cycle inhibition was generated by activation of p21 and reduction of the cyclin D-Cdk4 and cyclin E-Cdk 2 expression following animals' treatment with TNP-470. These results suggest that TNP-470 may be efficient for anti-angiogenic therapy and treatment of human HCC^[56].

p27

The p27 (p27 Kip1), whose gene, a member of haploinsufficient tumor suppressor gene family^[57], locates on chromosome 12p13.1-p12, is a cyclin-dependent kinase inhibitor. Cell cycle progression at G1 and cyclin E-CDK2 and cyclin D-CDK4 complexes are regulated by p27. Different studies have been executed to understand the role of p27 in development of tumor and particularly in HCC. The comparison of hepatocellular HCC with adjacent non-tumoral and normal liver tissues found that the weak expression of p27 was strongly associated with infiltration, metastasis and poor prognosis in patients affected by HCC. Moreover, cytoplasmic sequestration of p27 was observed more in HCC leading a diminution of p27 expression and was particularly characterized in early steps of hepatocarcinoma development^[58]. Philipp-Staheli and co-workers have already suggested that the p27 protein might be a check point for tumor suppression and

an important prognostic marker because its loss favors tumor growth^[59]. Expression of p27 was lower in patients having liver cirrhosis and HCC in comparison to those without HCC level^[60]. This group explained the loss of p27 expression by high level of methylation of its promoter. The high and low levels of p27 expression have been associated with prolonged survival and poor prognosis, respectively^[61,62]. A study proposed that functional inactivation of p27 is strongly associated with methylation of p16 and loss of its expression^[63].

p16

The p16 (P16INK4) gene, located on chromosome 9p21, encodes a protein that inhibits proliferation of normal cells by binding strongly with cdk4 and cdk6. This binding prevents cdk4 and cdk6 from interacting with cyclin D and inactivation by phosphorylation of the retinoblastoma protein (Rb). In 1998, post-transcriptional regulation was found to inactivate the p16 activity in HCC and this inactivation appeared to take place during the early-stage of hepatocarcinogenesis^[50]. The loss of expression of p16 and inactivation of Rb represent major events in hepatocarcinogenesis^[64]. Analysis of different hepatocyte cell lines revealed that increased p16 expression is associated with decreased phosphorylation of pRB. Reciprocally, phosphorylation of Rb and increase of cell growth were associated with silencing of p16 by promoter methylation^[65]. The p16 gene is silenced by hypermethylation of 5'CpG islands in its promoter^[66,67]. In addition to hypermethylation-induced transcriptional repression of the p16 gene, expression is also affected by mutation and deletion, although these modifications are less common^[38]. As described above, aflatoxin inactivates p53. The concentration of aflatoxin B1 albumin adducts has been correlated not only with p53 mutation but also with p16 methylation stressing the importance of environmental factors in the development of HCC^[68].

WNT PATHWAY

Many different proteins have been described in the Wnt pathway which function either as oncogenes (e.g beta-catenin) or as tumor suppressors (e.g E-cadherin, APC, Axin 1 and 2 proteins). About 50% of the HCC exhibited alteration of the Wnt pathway^[69].

E-cadherin

E-cadherin protein, encoded by a gene located on chromosome 16q22.1, belongs to the cadherin superfamily and is a calcium dependent cell-cell adhesion glycoprotein. Loss of function induced by mutations was associated with proliferation, invasion and metastasis. Many investigations with animal models and human HCC tissues have been performed and show that E-cadherin gene expression is regulated by promoter methylation. Hypermethylation was associated with decreased E-cadherin expression but also with microvascular invasion and recurrence of HCC^[70,71]. HBV and HCV affect this pathway. The presence of the HBx protein was associated with hypermethylation of the E-cadherin promoter, loss of its expression and beta-

catenin accumulation^[72]. In HCV-associated HCC the probability of recurrence could be linked to depressed E-cadherin expression^[73].

Axin1/ axin2

Axin1 and axin2 proteins, encoded by genes located on chromosome 16p13.3 and 17q23-q24, respectively, act as negative regulators of the Wnt signaling pathway and can induce apoptosis. Axins interact with different proteins such as beta-catenin, adenomatosis polyposis coli (APC) and glycogen synthase kinase 3-beta. Mutations in the Axin1 gene have been associated with different human cancers including HCC and hepatoblastomas. Satoh and co-workers identified mutations in the axin1 gene as well as in cell lines as in HCCs and they reported that wild type protein stimulates apoptosis leading to suppression of tumor growth. The gene coding for Axin1 protein is affected by loss of heterozygosity and small deletions, mutations and by missense mutations (1 bp deletion and 12 bp insertions)^[74] which most of the time target the gene in a biallelic manner^[75]. Combination of loss of heterozygosity and mutation induced the inactivation of Axin1 in HCC^[76]. The loss of heterozygosity also frequently affects the Axin2 gene due to its chromosomal localization and has been associated with different tumors like breast cancer and neuroblastoma^[77]. The percentage of Axin1 and Axin2 mutations in HCC remains controversial. In presence of Axin mutations, the Wnt-signaling pathway is altered leading to the accumulation of beta-catenin^[78-80]. Beta-catenin mutations were associated with over-expression of G-protein-coupled receptor (GPR) 49, glutamate transporter (GLT)-1 and glutamine synthetase (GS) while this correlation was not found with Axin1 mutations. These results suggest that Axin1 function might affect other signaling pathway than Wnt pathway^[76].

APC

A gene located on chromosome 5q21-q22 encodes a tumor suppressor protein named adenomatosis polyposis coli (APC). This protein has many intracellular functions including nuclear export and degradation of beta-catenin. A small region designated the mutation cluster region regroup mutations associated to diseases. To determine the role of APC protein in hepatocyte carcinogenesis, Colnot and co-workers generated a knock-out mouse model targeting exon 14 of the APC gene. They observed that 67% of analyzed mice developed HCC while Wnt/beta-catenin pathway activation was demonstrated by accumulation of different genes regulated by beta-catenin (leukocyte cell-derived chemotaxin 2, ornithine aminotransferase and glutamine synthetase, glutamate transporter 1)^[81]. The disruption of APC gene in liver induces hepatocyte hyperplasia, marked hepatomegaly and rapid mortality. Like other tumor suppressor genes, APC is hypermethylated in HCC relative to non-tumor liver. Hepatitis C virus/hepatitis B virus-negative HCC showed less methylation on APC gene than in hepatitis C virus-positive HCC^[82]. Methylation status of APC and other tumor suppressor genes was associated with the epigenetic instability dependent HCCs^[83]. Recently, bi-

allelic inactivation and nonsense mutation at codon 682 of APC gene in sporadic nodule-in-nodule-type HCC were observed using high-density array-based comparative genomic hybridization (aCGH) and direct sequencing, respectively. Both alterations lead to inactivation of APC binding to beta-catenin which may enhance the evolution of sporadic HCC^[84].

RAS/JAK/STAT PATHWAY

SOCS

The suppressor of cytokine signaling (SOCS), also known as STAT-induced STAT inhibitor (SSI) protein family comprises several members including SOCS1, SOCS2 and SOCS3 which are encoded by genes located in 16p13.13, 12q, 17q25.3, respectively^[85,86]. These proteins function as negative regulators of cytokine signaling. SOCS1 as well as SOCS2 and SOCS3 are stimulated by cytokines and act in negative feedback loop to regulate cytokine signaling. SOCSs inhibit by direct binding the kinase activity of Janus Kinases (JAKs) proteins and so block the JAK/STAT pathway^[87]. The role of SOCS1 in negative regulation of interferon-gamma and in T-cell differentiation was determined by generating a knock-out mouse model lacking the expression of SOCS1 gene. In the same way, SOCS2 and SOCS3 were demonstrated to be involved in regulation of postnatal growth and regulation of fetal liver hematopoiesis, respectively^[87]. The SOCS1 gene promoter is enriched with CpG dinucleotides^[88]. The decrease of SOCS1 expression was associated with aberrant methylation in CpG islands and 5'non-coding region of SOCS1 promoter and loss of heterozygosity^[89,90]. A significant relationship between SOCS1 methylation level and HCC transformation of cirrhotic nodules was established confirming that SOCS might act as a tumor suppressor^[91]. The suppression of cell growth and activation of JAK2 was observed after recovering of SOCS1 expression by gene therapy in cells having SOCS1 silenced by hypermethylation^[92]. SOCS3 promoter can also be methylated resulting in diminution of SOCS3 expression^[93,94]. Restoration of its expression blocks the phosphorylation of STAT3 and inhibits the proliferation. Cell growth and migration were negatively regulated by inhibition of JAK/STAT signaling pathway by SOCS3 protein in HCC^[93]. The enhancement of proliferation and development of hepatic tumor, activation of STAT3 and inhibition of apoptosis were observed in absence of SOCS3 expression obtained using a conditional knockout mice approach under carcinogenic condition. These results confirmed that SOCS3 acts as tumor suppressor gene^[95]. Leong and co-workers found that SOCS2 and SOCS3 were both up-regulated in hepatic cells following estrogen treatment *via* estrogen receptor (ER) alpha providing a mechanistic explanation for the rare cases of HCC responding to this treatment^[96].

RASSF1A/NORE1

Members of the RAS superfamily are plasma membrane GTP binding proteins that modulate intracellular signal transduction pathways. A subgroup in this family contains

a Ras-association domain and takes part in RAS signaling pathway^[97-101]. A gene located on chromosome 3p21.3 encodes a protein identified as human RAS effector homologue (RASSF1). Alternative splicing and promoter usage of this gene generates three different transcripts: RASSF1A, RASSF1B and RASSF1C. RASSF1A contains a Ras Association Domain (RA) and binds Ras in a GTP-dependent manner to affect apoptosis. The presence of CpG-islands in the promoter of the RASSF1 gene was associated with high methylation level and the loss of expression of RASSF1A. The reduction of RASSF1A expression was found in lung carcinoma and these results indicate that RASSF1A might act as a tumor suppressor^[102]. Additionally, RASSF1A role is associated with the cell cycle given that it blocks the accumulation of cyclin D1 and G (1)/S-phase cell cycle progression^[103]. Hypermethylation of RASSF1A induced the inactivation of RASSF1A but also correlated with environmental carcinogens such as AFB (1) and with inactivation of p16INK4a protein in hepatocellular carcinoma^[104]. The high frequency of hypermethylation of RASSF1A promoter could be detected not only in tumor^[83,105] but also in matched plasma of patients affected by HCC^[105]. Methylation level of RASSF1A was proposed as a potential marker to diagnose HCC^[100,106,107]. Recently, a strong association was identified between CpG island methylation phenotype (CIMP) and serum concentration of alpha-fetoprotein (AFP) and inactivation of many genes involved in process of tumor suppression such as RASSF1A. Thus CIMP might be used as molecular marker of late-stage HCC development^[12].

Among the subfamily of RAS effectors, the NORE1 proteins encoded by a gene located on chromosome 1q32.1 were described as a regulator of Ras dependent apoptosis. In the same manner as the RASSF1 gene, three different isoforms were identified (NORE1Aalpha, NORE1Abeta and NORE1B). NORE1A and NORE1B, which are separated by CpG islands spanning their first exons, share the Ras-association (RA) domain but the diacylglycerol (DAG) binding domain was only present on NORE1A^[99]. Analysis of methylation found that NORE1A promoter was methylated whereas NORE1B was unmethylated in breast, colorectal and kidney tumor cell lines^[97,99]. Comparative analysis showed that NORE1 gene was not altered by methylation whereas RASSF1A gene promoter was increasingly methylated from regenerating liver to hepatocellular carcinoma nodules^[107]. In another study, expression of NORE1A and SOCS 3 was inhibited by high methylation level of their promoters and their inactivation associated with a subclass of poor prognosis HCC^[94]. It appears that NORE1 could be considered as tumor suppressor gene in hepatocarcinogenesis^[108].

OTHER PATHWAYS

KLF6

Krüppel-like factors (KLFs) are highly related zinc-finger proteins that are important components of the eukaryotic cellular transcriptional machinery. Among this protein family, Krüppel-like factor 6 (KLF6) is a ubiquitously expressed zinc finger transcription factor

encoded by a gene located on chromosome 10p15^[109]. The KLF6 protein binds DNA on guanine-rich core promoter elements and regulates the transcriptional activation process *via* its zinc fingers domains and central acidic N-terminal domain. Due to its regulatory role in transcription, different studies have been conducted to define the role of KLF6 in tumor development and has been described as a tumor suppressor in different cancer such as colon and prostate^[110-112]. Reduction of KLF6 expression and methylation of KLF6 promoter have been associated with human cancers^[111,113-115] but the incidence of KLF6 variation on HCC has been described for first time by Friedman's group^[116]. They determined that loss of heterozygosity induces a loss of KLF6 expression in 50% of analysed HCC samples. In same samples, they identified several missense mutation associated with presence of HBV or HCV. Moreover, *in vitro* analyses of KLF6 mutations showed that only KLF6 wild type inhibits the cell growth of HepG2 cell lines by p21 protein activation whereas the different mutants did not induce any changes indicating regulatory effects of mutations on KLF6 activity. The process leading to the activation of p21 and reduction of cell proliferation by KLF6 necessitates the acetylation of KLF6 by histone acetyltransferase activity of either cyclic AMP-responsive element binding protein-binding protein or p300/CBP-associated factor. This process can be abrogated by a single mutation of lysine-to-arginine (K209R), point mutation already described in prostate cancer^[117]. In recent work carried out on ovarian cancer, E-cadherin level variation was found to be mediated by direct action of KLF6 on its promoter^[118]. Furthermore, induction of cellular differentiation and inhibition of cell proliferation was observed in KLF6 overexpressing HepG2 cell lines and associated with augmentation of E-cadherin and albumin expression and reduced cyclinD1 and beta-catenin expression. In the same study, the authors demonstrated also inhibitory effects of HBV and HCV infection on KLF6 expression^[119]. The stability of KLF6 is modified by ubiquitination after induction of apoptosis by drugs (cisplatin, adriamycin) or UVB irradiation but not by apoptotic-dependent extrinsic/death-receptor pathway. The effects of KLF6 on tumor suppression and enhancement of chemotherapeutics response might be affected by the speed of its degradation and deregulation of its stability^[120]. Apoptosis induced by upregulation of p53 and diminution of Bcl-xL expression was enhanced following KLF6 knockdown. Additionally, the arrest of cell cycle in G1 phase and expression of cyclin-dependent kinase 4 and cyclin D1 were weakened or suppressed in KLF6 silenced cells. These results bring a new perspective for the link between of KLF6 and apoptosis^[121].

PTEN

This haploinsufficient gene located on chromosome 10q23.3 encodes a phosphatidylinositol-3,4,5-trisphosphate 3-phosphatase^[122]. This protein dephosphorylates phosphoinositide substrates and acts as a negative regulator for intracellular levels of phosphatidylinositol-3,

4, 5-trisphosphate. PTEN is inactivated in many cases of breast, endometrial, prostate cancers. Many mutations affect PTEN gene such as missense mutations in exon 5 (K144I) and exon 7 (V255A) or silent mutations in exon 5 (P96P) in HCC from Taiwan^[123]. In HBV infected liver cells, PTEN expression is also deleted following the genome integration of HBV^[124]. In this Asiatic subset of HCCs, both mRNA and protein levels of PTEN were weaker in tumor than in paired para-carcinoma tissues^[125,126]. Moreover, a point of mutation induced a weak level of PTEN which is inversely linked with FAK phosphorylation^[127]. In 47% of HCC analyzed by Sieghart and co-workers, they observed a decrease or absent of PTEN which is inversely correlated with expression of phosphorylation proteins in mTOR pathway^[128]. HCC development might be susceptible to inhibition of mTOR^[129]. The lessening of PTEN expression was associated with loss of promoter activity^[130]. Wang and collaborators confirmed that PTEN inactivation seems resulting not only of mutation and promoter methylation but also possible other epigenetic mechanisms which remain to be defined^[131]. Reduction of PTEN expression in HCC was associated with poor prognosis and progression of HCC^[132]. This might be due to the inverse correlation between PTEN expression and VEGF expression^[133].

HINT protein

Hint1: Hint1 is encoded by gene located on chromosome 5q31.2 and is member of the The Histidine Triad (HIT) protein family characterized the His-X-His-X-His-XX motif. Hint1 forms homodimers and each subunit can bind a nucleotide. Hint1 acts as an adenosine 5'-monophosphoramidate (AMP-NH₂) hydrolase^[134]. Spontaneous immortalization and enhancement of cell growth were observed in cells lacking Hint1. Squamous tumors (both papillomas and carcinomas) of the forestomach developed after treatment with chemical carcinogen N-nitrosomethylbenzylamine (NMBA) showed a greater volume and a more severe degree of malignancy in Hint1-/- mice^[135]. Accordingly, the hypothesis of Hint1 role as tumor suppressor was developed and work was carried out to elucidate which signaling pathway is involved. Weiske and co-workers determined an interaction between Hint1 and pontin and reptin^[136]. Pontin and reptin are often found in complexes and they possessed single-stranded DNA-stimulated ATPase and ATP-dependent DNA helicase activity but with opposite action^[137,138]. Moreover, both proteins interact with beta-catenin by modulating its transcriptional activity in Wnt pathway^[139]. Hint1 was identified as a part of the LEF-beta-catenin transcription complex and function as a negative regulator of TCF-beta-catenin transcriptional activity, repressing expression of Wnt signaling target genes such as axin2 and cyclin D1^[136]. The same authors reported that p53-induced apoptosis was influenced by Hint1, which up-regulated Bax and down-regulated Bcl-2. The pro-apoptotic activity of Hint1 was not related to its enzymatic AMP-NH₂ hydrolase activity^[140]. Treatment of non small cell lung cancer (NSCLC) cell line with DNA demethylating agent, 5-aza-2'-deoxycytidine up-regulated of Hint1, and this correlated with growth inhibition and reduction of

tumorigenicity^[141]. Weinstein's group described Hint1 is a novel haploinsufficient tumor suppressor gene and is able to repress the cell growth and tumor progression by inhibition of AP-1 transcription factor activity in mammary tumor and colon cancer cells, respectively^[142,143]. The role of Hint1 in hepatocarcinogenesis remains to be explored.

Hint2: Hint2 is a mitochondrial HIT protein. This protein is encoded by a gene located on chromosome 9p13.3. Tissue profile expression of Hint2 showed that this protein is predominantly expressed in the liver and pancreas. Like its cytoplasmic homologue Hint1, Hint2 acts as an adenosine monophosphate hydrolase enzyme and this enzymatic activity was lost when the second histidine of the HIT motif is mutated. The sensitivity of cells to apoptosis was increased when Hint2 was over expressed whereas Hint2 knockdown was coincident with reduced caspase 3 expression. Subcutaneous injection of HepG2 cells over-expressing int2 in SCID mice resulted in maller tumours, which displayed more apoptosis in comparison to mice, injected with control HepG2 cells. Microarray analyses carried out on human tissues found a significant reduction of HINT 2 mRNA in HCC compared with surrounding liver tissue. This diminution of Hint2 expression was associated with a poor prognosis^[144].

FRAGILE CHROMOSAL SITE GENES AND HCC

FHIT

The FHIT protein is encoded by a haploinsufficient tumor suppressor gene^[145], located on chromosome 3p14.2 a place known to be one of the most fragile sites in human genome. This protein, a member of the histidine triad gene family, is a diadenosine triphosphate hydrolase. In numerous tumor types such as lung, stomach, breast, colon, aberrant forms of FHIT protein were found due to rearrangements and deletions in region of FHIT locus^[146-148]. Aberrant FHIT transcripts with deletions of exons and fusion of remaining exons and loss of heterozygosity were observed in HCC tissues in comparison with non-tumoral tissues and lead to the lack of FHIT protein expression. So FHIT was frequently altered in liver and might be implicated in liver tumorigenesis^[149-151]. FHIT has also been described as a pro-apoptotic agent. Restoration of Fhit expression activated caspase 8 and induced apoptosis in Fhit-negative cell lines^[152]. Sard reported a 2-fold increase in the apoptosis-related protein Bak and in the cell cycle inhibitory protein p21, but not in Bcl-2, Bcl-X, Bax and, p53^[153]. Inactivation like by promoter methylation was associated with progression and poor prognosis of HCC^[154,155]. In 2004, a study performed on HCC cohort from the US found over-expression of modified FHIT transcripts^[156]. Fusion between exon 5 and 7 and between exon 7 and 9 were frequently observed in HCV-related^[157]. Apoptosis was weaker in early HCC with negative FHIT expression than in advanced HCC with positive expression of FHIT. So, the absence of FHIT protein influencing the

balance between apoptosis/proliferation may play a role in formation of HCC^[158].

WWOX

WWOX (WW-domain containing oxidoreductase) gene located on another fragile site on chromosome 16q23.3-q24 encodes a 46 kDa protein having 2 WW domains, which mediate the protein-protein interaction, and a short-chain dehydrogenase/reductase domain (SRD). The WW domain-proteins are expressed in all eukaryotes and act as regulator of a wide variety of cellular functions such as RNA splicing, transcription and protein degradation. Different forms of WWOX transcripts were observed following deletions or alternative splicing in frameshift leading to the total or partial loss of different domains. Diverse missense mutations and single nucleotide polymorphisms (SNP) within the WWOX have been identified in many tumor cell lines and conduct to consider this protein as a tumor suppressor^[159]. No mutations leading to WWOX abnormal transcript were found in HCC cell lines. However, loss of heterozygosity on chromosome 16q at the fragile site FRA16D was found in 29% of HCC and an association between 16q lack and R249S mutation affecting the p53 gene was determined in HCC samples from patients exposed to aflatoxin B1^[160]. Decreased or absent expression of WWOX was observed in cell lines derived from human HCCs^[161]. WWOX protein is able to interact with other proteins, in particular with p73 protein^[162,163]. The association of WWOX with p73 redistributes p73 from nucleus to cytoplasm blocking p73 transcriptional activity and enhancing the pro-apoptotic potential of WWOX^[164]. A recent study performed on mouse models treated with carcinogen drugs found that the same down regulation profile for FHIT and WWOX in the liver^[80]. This result completes the association between FHIT and WWOX expression observed in breast and gastric cancer^[164-167].

Parkin (PARK2)

Like WW domain-containing oxidoreductase gene (WWOX; 16q23) and fragile histidine triad gene (FHIT; 3p14.2) parkin is also located on fragile and unstable chromosomal region (FRA6), which can be targeted by mutational rearrangement (duplication or deletion). Parkin is a member of RBR protein family implicated in ubiquitin related-proteolytic pathway^[168-170]. This protein was involved in neurodegenerative diseases^[171]. Parkin protects neurons and autosomal recessive juvenile parkinsonism is associated with mutations affecting this gene. Parkin seems to play a role in tumor suppression^[172]. Analysis of HCC samples has been performed by Wang and collaborators. They determined that the expression of parkin was lower in HCC compared with normal liver. Transfection of Hep3B cells with parkin increased their sensitivity to apoptosis and negatively regulated their cell growth. These results prompt speculation that the absence of parkin expression may favor the development of HCC^[173]. Down-regulation and loss of parkin expression was correlated with methylation of its promoter in acute lymphoblastic leukemia and chronic myelogenous leukemia (CML) in lymphoid blast crisis^[174].

NEW POTENTIAL PATHWAYS IN TUMOR SUPPRESSION

DNA methyltransferase

A family of protein called DNA methyltransferase catalyses the methylation process on CpG islands leading to regulation of gene expression. Two distinct gene families: DNMT1 gene and DNMT3 gene family which regroups two related genes: DNMT3a and DNMT3b are known to maintain and induce the methylation. The function of DNMT2 is still to be clarified^[175-177]. High mRNA levels of DNMT1 and DNMT3 were observed in HCC in comparison of normal or non-tumoral tissues but only cytoplasmic DNMT3 expression was decreased in HCC. The first studies proposed that these proteins act during early stage of hepatocarcinogenesis and might take a part in progression of HCC^[178-180]. Excepted for a correlation between DNMT3a immuno-reactivity and p53 methylation levels, Park and co-worked did not observed a correlation between DNMT expression and methylation levels of different tumor suppressor genes described in HCC^[11]. It seems that not only DNMTs protein but also other mechanisms are engaged in methylation changes of tumor suppressor genes in HCC. In liver cell line infected by HBV and HBV-infected HCC samples, HBVx proteins induced a change of transcription of DNMTs proteins leading to regional methylation of tumor suppressor genes^[181]. The lack of two other proteins: O6-Methylguanine-DNA Methyltransferase (MGMT) and human Mut L homologue (hMLH) implicated in DNA repair system have been associated with poor prognosis and advanced stages of HCC^[182]. The expression of MGMT in HCC is altered by hypermethylation of its promoter and DNA integration of HBV near to FRA10F chromosome fragile site^[183,184].

miRNA and HCC

Small non-coding RNAs 19 to 25-nucleotide-long RNA called microRNAs (miRNA) define a new family of regulatory molecules. They recognize and bind the complementary sequences in 3' untranslated regions (3'-UTR) of diverse target mRNAs^[185]. By their role in control of diverse cellular processes such as proliferation, differentiation and apoptosis, miRNAs appear as new actor of regulation of tumorigenesis. However, miRNAs are themselves the target of regulation and their expressions are down- or up-regulated in various human cancer types and they can act as tumor suppressors or oncogenes^[186-192]. Murakami and collaborators identified five mRNAs (miRNA 199a, 199a*, 200a, 125a and 195) and three (miRNA 224, 18, p18) with lower and higher expression in HCC than in adjacent non-tumoral tissues, respectively^[184]. Kutay and Gramantieri observed that miR-122a, the most represented miRNA in liver, is down regulated in HCC^[193,194]. Moreover, Gramantieri and collaborators showed a reverse correlation between miR-122a and cyclin G1. This miRNA appears to block the tumor growth through the inhibition of cyclin G1 expression^[194]. miR-122a has been also described to facilitate the replication of HCV RNA in HCC^[195]. The genomic integration of HBV

in region of fragile site alters the expression of miRNA^[196]. In fact, the genome integration of HBV in particularly in fragile site alters the expression of different miRNA. The expression of let-7e is lower in HCC than in non tumor liver and this inhibition is a consequence of HBV integration in FRA11B, FRA11G and FRA19A fragile sites^[184,187,196]. miRNA-195 which modulates the expression of Bcl-2 like protein, SKI oncogenes and methyl-CpG binding protein-2 in HCC, is altered by integration of HBV in FRA17A^[184,196].

CONCLUSION

Numerous proteins involved on the control of different cellular processes such as cell proliferation, apoptosis and DNA replication are described as tumor suppressor. The deregulation of expression of these proteins by mutations and/or methylation of their promoter and viral-dependent-action contributes to the progression of hepatic cancers. Comprehensive understanding of the functions of these tumor suppressors is a prerequisite to devise innovative treatments of HCC.

DEFINITIONS

Tumor suppressor

Cell fate is controlled by division, differentiation and death. The balance between these commitments is determined by negative and positive regulations mainly through two classes of genes: the tumor suppressor genes and the proto-oncogene genes, respectively. In normal condition, tumor suppressor genes repress the formation and development of tumor but damage in their expression or function conduct to uncontrolled cell growth or cancer. Their function is impaired by mutations, loss of chromosome region or silencing by promoter methylation. Tumor suppressor genes are important targets in the quest to develop clinical therapies based on the restoration of gene function to reverse the carcinogenesis process.

Haploinsufficiency

The majority of tumor suppressor genes follow the "two-hit hypothesis". The loss of function is associated with a damage of both alleles. In case of haploinsufficiency, the missing of only one allele is sufficient to inactivate the synthesis of gene product and to confer in a dose-dependent manner tumor sensitivity. Haploinsufficiency is associated with a few tumor suppressor genes such as p53 and PTEN.

Methylation state

Expression of genes can be regulated by methylation of their promoter. DNA methylation is the conversion of cytosine to 5-methylcytosine, which is catalyzed by DNA methyltransferase. It occurs on CpG sites mainly located in promoter region of genes. In case of cancer, aberrant methylation affects several tumor suppressor genes such as E-cadherin and SOCS1 leading to gene silencing and loss of protein function. The simultaneous methylation of CpG islands of multiple genes defines a new biomarker

named CpG island methylator phenotype (CIMP). CIMP has been recognized as an important mechanism of gene regulation.

Loss of heterozygosity (LOH)

Loss of heterozygosity (LOH) refers to the loss of a single allele of a gene due to mutation, deletion of large chromosome segment and epigenetic regulating events such as methylation. LOH frequently affects tumor suppressor genes because most of them are located in chromosome fragile site. Losses in regions 1p, 4q, 6q, 8p, 13q, 16q, and 17p have been related to HCC and induce the lack of gene such as E-cadherin, WWOX, FHIT or p53.

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TOPIC HIGHLIGHT

Harry HX Xia, PhD, MD, Series Editor

Molecular mechanism underlying the functional loss of cyclindependent kinase inhibitors p16 and p27 in hepatocellular carcinoma

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Abstract

Hepatocellular carcinoma (HCC) is one of the most common human cancers, and its incidence is still increasing in many countries. The prognosis of HCC patients remains poor, and identification of useful molecular prognostic markers is required. Many recent studies have shown that functional alterations of cell-cycle regulators can be observed in HCC. Among the various types of cell-cycle regulators, p16 and p27 are frequently inactivated in HCC and are considered to be potent tumor suppressors. p16, a G1-specific cell-cycle inhibitor that prevents the association of cyclindependent kinase (CDK) 4 and CDK6 with cyclin D1, is frequently inactivated in HCC *via* CpG methylation of its promoter region. p16 may be involved in the early steps of hepatocarcinogenesis, since p16 gene methylation has been detected in subsets of pre-neoplastic liver cirrhosis patients. p27, a negative regulator of the G1-S phase transition through inhibition of the kinase activities of Cdk2/cyclin A and Cdk2/cyclin E complexes, is now considered to be an adverse prognostic factor in HCC. In some cases of HCC with increased cell proliferation, p27 is overexpressed but inactivated by sequestration into cyclin D1-CDK4-containing complexes. Since loss of p16 is closely related to functional inactivation of p27 in HCC, investigating both p16 and p27 may be useful for precise prognostic predictions in individuals with HCC.

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Key words: Hepatocellular carcinoma; Cell-cycle regulator; Cyclin-dependent kinase inhibitor; DNA methylation; DNA methyltransferase; p16; p27; FoxM1b

INTRODUCTION

The liver is a remarkable organ, since it can regenerate after significant tissue loss. When liver tissue is partially lost or injured, residual hepatocytes immediately enter the cell cycle from a quiescent (G0) state through pre-replicative (G1), DNA synthesis (S) and mitosis (M) states. Significantly, once the original volume of the liver is achieved, the hepatocytes revert to their quiescent state and restore the tissue volume to a physiological condition. This exquisite regenerative potential of the liver has been regarded as potentially being governed by sequential activation of a series of positive and negative cell-cycle regulators in hepatocytes. Briefly, cell-cycle entry is mainly promoted by cyclins A, D and E with cyclin-dependent kinase (CDK) 2 and 4 and the activities of these CDKs are negatively regulated by the cyclin-dependent kinase inhibitors (CDKIs) p16 and p27 to avert excessive hepatocellular replication. Therefore, it is understandable that many oncologists have sought to elucidate the molecular mechanism underlying cell-cycle regulators during hepatocarcinogenesis^[1,2].

To date, many studies have reported genetic and epigenetic alterations of cell-cycle regulators in hepatocellular carcinoma (HCC). In particular, both p16 and p27 are frequently altered in HCC, suggesting that they are potent tumor suppressors. Of note, epigenetic alterations of p16 in liver cirrhosis have recently been reported^[3-5], supporting the idea that p16 may be involved in the early stage of hepatocarcinogenesis. Moreover, we and others have found a close relationship between p16 and p27 during the progression of HCC^[6-8], indicating that comprehensive analyses of these CDKIs may

be valuable for further understanding of the clinical significance of cell-cycle regulators in HCC. In this review, an overview of the molecular mechanism underlying p16 and p27 inactivation in HCC is presented, and the clinical significance of the relationship between these CDKIs in individuals with a high risk of HCC is described.

FREQUENT INACTIVATION OF p16 IN HCC

p16 is a specific inhibitor of CDK4 and CDK6 and potentially blocks G1 cell-cycle progression *via* dephosphorylation of Rb through its inhibitory effect on CDK4/cyclin D1 complex activity^[9,10]. The *CDKN2/MTS1* gene encoding p16 is frequently deleted or inactivated by 5'-CpG island hypermethylation in various types of cancer cells, and p16 is considered to be a potent tumor suppressor^[10,11]. Many studies have focused on searching for the genetic/epigenetic status of p16 in HCC with a view to investigating the molecular mechanism of hepatocarcinogenesis. Biden *et al* reported that p16 was frequently mutated or deleted in human HCC in an Australian population^[12], while Chaubert *et al* reported germline mutations of p16 in a subset of familial HCC in Switzerland^[13] and Piao *et al* reported the presence of a homozygous deletion of p16 in 61% of HCC cases in a Korean population^[14]. On the contrary, we examined the genetic status of p16 in 60 HCC cases in Japan but did not find any homozygous loss of *p16*^[15]. Only 4 of 60 (6.6%) HCC cases showed intragenic mutations in exon 2 of *p16*, and no hotspots for amino acid changes were detected in our study. Significantly, however, we found that the p16 gene promoter was methylated in 27 of 60 (45%) HCC cases. A close relationship between loss of p16 immunostaining and hypermethylation of the p16 gene was found, indicating that epigenetic alterations induced loss of p16 protein expression in HCC^[15].

Interestingly, 24 of 60 (40%) HCC cases showed high levels of methylation in the p16 gene promoter region, ranging from 60%-85% of the CpG islands as assessed by methylation-sensitive single nucleotide primer extension^[16]. The reason why the patterns of p16 inactivation differ among the studies is unclear. It should be noted that Li *et al* reported that HCCs with *p16* methylation were only found in individuals with hepatitis B virus (HBV) or hepatitis C virus (HCV) infection and not in virus-negative individuals^[4]. Since many other studies have found rare homozygous deletions and infrequent mutations of *p16* in HCCs^[17-19], epigenetic changes may be the main reason for p16 inactivation in hepatitis virus-associated HCC, while genetic mutations of p16 may occur in specific geographical conditions and pedigrees.

MOLECULAR MECHANISM OF p16 DNA METHYLATION IN HCC

The molecular basis of the aberrant hypermethylation of CpG islands of p16 observed in many cases of HCC is unknown. One possible mechanism is upregulation of DNA (cytosine-5)-methyltransferase (DNMT), which is widely assumed to be responsible for most of

the methylation of the human genome. Unfortunately, however, the relationship between DNA methyltransferase and gene methylation in cancer cells is controversial. For example, Eads *et al* reported that deregulation of DNA methyltransferase gene expression in human colorectal cancer cells did not play a role in establishing tumor-specific abnormal DNA methylation patterns^[20]. Furthermore, Rhee *et al* reported that the p16 gene remained fully methylated and silenced in colorectal carcinoma cells lacking DNMT1, while genetic disruption of both DNMT1 and DNMT3b nearly eliminated methyltransferase activity and reduced genomic DNA methylation by more than 95%, indicating that cooperation of these two enzymes may be essential for maintaining DNA methylation in cancer cells^[21,22]. In contrast, Robert *et al* reported that specific depletion of DNMT1, but not DNMT3a or DNMT3b, markedly potentiated the ability of the demethylating agent 5-aza-2'-deoxycytidine to reactivate silenced tumor-suppressor genes^[23], suggesting that DNMT1 may be sufficient for maintaining CpG island methylation in human cancer cells.

Histone deacetylation is another possible mechanism of p16 methylation, since DNA methylation and repressive chromatin characterized by histone deacetylation appear to act as synergistic layers for the silencing of global genes. However, the molecular relationship between p16 and histone deacetylation is not clear. Cameron *et al* reported that hypermethylated genes, including p16, cannot be transcriptionally reactivated with trichostatin A (a specific inhibitor of histone deacetylase)^[24]. Zhu *et al* reported that p16 repressed in human lung cancer cells was induced by synergistic cooperation of depsipeptide (an inhibitor of histone deacetylase) and 5-aza-2'-deoxycytidine^[25]. Interestingly, however, they also reported that cells treated with higher concentrations of 5-aza-2'-deoxycytidine and depsipeptide showed decreased p16 expression together with significant suppression of cell growth^[25]. These lines of evidence indicate that the molecular mechanism of p16 DNA methylation in cancer cells may vary among different types of cancer cells.

It is noteworthy that Guan *et al* reported a close relationship between K-ras mutations and p16 methylation in colon cancer, by showing that a K-ras-transformed colon cancer cell line exhibited increased DNA methyltransferase activity and p16 gene methylation^[26]. In HCC, Weihrauch *et al* reported that frequent K-ras mutations and p16 methylation were found in vinyl chloride-induced HCC^[27], suggesting that K-ras mutations may be important events in p16 inactivation in chemically-induced HCC. Although there have been no reports of the relationship between hepatitis virus infection and K-ras mutation, these reports may shed new light on why p16 is inactivated in many cases of HCC. A thorough examination of the relationship between p16 methylation and K-ras mutations in HCC is awaited.

p16 GENE IS METHYLATED DURING THE EARLY STEPS OF HEPATOCARCINOGENESIS

Several recent studies have revealed that *p16* is methylated in some sets of individuals with non-HCC liver tissues

associated with chronic hepatitis virus infection. Kaneto *et al* reported that *p16* methylation was detected in 5 of 17 (29.4%) cirrhosis patients and 4 of 17 (23.5%) chronic hepatitis patients, all of whom were associated with HBV or HCV infection, but not in normal liver and other non-viral liver diseases^[3]. Similarly, Li *et al* reported that the *p16* gene was methylated in 6 of 38 (16%) HBV-infected or HCV-infected chronic hepatitis and cirrhosis patients bearing HCC^[4]. We found that *p16* was methylated in 4 of 112 (4%) liver cirrhosis patients^[5], all of whom were infected with HCV (personal communication). Although the ratio of *p16* DNA methylation in non-HCC liver tissues observed in our study was low compared with previous studies, these lines of evidence suggest that hepatitis virus infections may play roles in the induction of *p16* promoter methylation. Very recently, Jung *et al* reported that HBV X protein induced DNA hypermethylation of the *p16* promoter to repress its expression and led to transcriptional activation of DNMT1 *via* the cyclin D1-CDK4/6-pRb-E2F1 pathway^[28], suggesting a close relationship between hepatitis virus infections and aberrant methylation of the *p16* gene. Therefore, examinations of the degree of *p16* DNA methylation in non-HCC liver tissues with hepatitis virus infections, by bisulfate sequencing for example, may be of use for further elucidating the roles of hepatitis viruses in *p16* inactivation in the liver.

CLINICAL SIGNIFICANCE OF *p16* IN HCC

To investigate the clinical significance of *p16* inactivation in HCC, we evaluated the labeling index (LI) of *p16* in HCCs by immunohistochemical staining and found that the proportion of tumors with negative staining increased as the histopathologic grading of the tumors tended to become more poorly differentiated. Similarly, Ito *et al* reported that the *p16* LI was significantly decreased in cases with advanced-stage HCC^[29]. However, regarding the prognosis of individuals with HCC, *p16* does not seem to be an independent risk factor. Tannapfel *et al* reported that they were unable to establish alterations of the *p16* locus as an independent prognostic factor for HCC^[30]. Anzola *et al* found no associations between *p16* inactivation and clinicopathological characteristics or prognosis^[31], and suggested that this may arise because *p16* potentially plays an important role in hepatocarcinogenesis and is frequently altered from the early stages of HCC. As described later, we recently found that the status of *p16* affects the prognosis of HCC with *p27* overexpression^[6], indicating *p16* may become a supportive prognostic factor in HCC when combined with other prognostic factors. In other words, *p16* may become an important therapeutic target for HCC. Although there have been little studies of the relationship between the level of CDK4/6 activities and *p16* inactivation in HCC patients, the fact that *p16* is widely inactivated in HCC does give us the idea that a clinical trial of CDK4/6-selective inhibitor in patients with HCC may be hopeful.

REGULATORY MECHANISM OF *p27*

p27 is a member of the KIP family of CDKs^[32,33], which negatively regulates the G1-S phase transition by inhibiting

the kinase activities of CDK2/cyclin A and CDK2/cyclin E complexes. During the cell cycle, the *p27* protein level is highest at G0/G1 phase and lowest at S phase, and is mainly regulated by protein degradation *via* a Skp1-Cullin-F-box protein (SCF)-type ubiquitin ligase complex that contains Skp2 as the substrate-recognizing subunit^[34,35]. It has also been found that Skp2 acts as the main rate-limiting regulator for *p27* degradation^[36-38] and that cyclin kinase subunit 1 (Cks1) is essential for efficient Skp2-dependent destruction of *p27*^[39,40].

p27 IS AN ADVERSE PROGNOSTIC FACTOR IN HCC

It is noteworthy that *p27* is widely regarded as an adverse prognostic indicator in many types of cancers, since decreased or absent expression of *p27* is frequently observed in cell nuclei in various types of human cancers with poor prognoses^[41]. In HCC, many studies have reported that decreased *p27* expression is closely associated with clinical invasiveness of the tumors. Ito *et al* reported that the *p27* LI was significantly decreased in cases with portal invasion, poor differentiation, larger size and intrahepatic metastasis among 104 HCC cases examined^[29], and suggested that *p27* can act as an independent predictor of HCC recurrence among several types of G1-S cell-cycle regulators (e.g., pRb, p21, p16, p53, cyclin D1 and cyclin E). Fiorentino *et al* examined 54 HCC cases and reported that high expression of *p27* was a favorable independent prognostic parameter^[42]. Tannapfel *et al* reported that *p27* was decreased in advanced cases in a series of curatively resected HCCs^[43]. Qin *et al* reported that longer disease-free survival rates were observed in patients whose tumors had higher *p27* (KIP1) expression^[44]. Armengol *et al* reported that *p27* constituted an independent predictor of recurrence after surgical resection in 46 cirrhotic patients with small HCCs^[45]. Zhou *et al* reported that the LI of *p27* was associated with differentiation, invasiveness and metastasis of tumors among 45 HCC cases examined^[46]. Similar to these previous reports, we found that HCCs expressing low levels of *p27* (low-*p27* expressers; *p27* LI of < 50% in the tumor cells), as evaluated by immunohistochemical staining, showed significantly favorable prognoses compared with individuals with HCCs showing high *p27* expression (high *p27* expressers; *p27* LI of > 50%). Kaplan-Meier survival curves revealed that the 5-year survival rates of the low-*p27* and high-*p27* expressers were 62% and 93%, respectively, and log-rank tests revealed that low *p27* expression was associated with a higher relative risk of dying from HCC than high *p27* expression^[6]. Taken together, *p27* can be regarded as a powerful clinical indicator for prognosis prediction in individuals with HCC.

p27 IS OVEREXPRESSED IN SOME SETS OF AGGRESSIVE HCCS

As described above, many studies have suggested that decreased *p27* expression can be regarded as a risk factor in individuals with HCC. Recently, however, extensive

studies have unraveled a novel aspect of the role of p27. Sganmbato *et al* reported that the levels of p27 were significantly increased in some breast cancer cell lines that exhibited exponential growth and high levels of cyclin D1 and cyclin E^[47]. Fredersdorf *et al* reported that p27 expression was increased in some cases of highly proliferative breast cancer cells overexpressing cyclin D1^[48]. Interestingly, Ciaparrone *et al* reported that a subset (35%) of colorectal carcinomas displayed diffuse cytoplasmic staining for p27 by immunohistochemical staining^[49], and Singh *et al* reported that cytoplasmic localization of p27 was associated with decreased survival in Barrett's associated adenocarcinoma^[50].

To comprehensively investigate the clinical significance of p27, we thoroughly examined the p27 status in HCC by immunohistochemical staining and found that the Ki-67 LI, a proliferation marker, varied from low to high among the high-p27 expressers (p27 LI of > 50%). Our results indicated that the tumors could be categorized into two groups according to a Ki-67 LI threshold of 20%, revealing that 26 of 40 (65%) high-p27 expressers had a Ki-67 LI of < 20% (2%-13%), while the remaining 14 (35%), including all 7 cases with cytoplasmic p27 immunostaining, had a Ki-67 LI of > 20% (22%-42%)^[6]. To address the reason for the different cell proliferation status among high-p27 expressers, the kinase activity of CDK2 was analyzed in HCC samples from the high-p27 expressers. The results revealed that the CDK2 activities were low in 8 of 12 (67%) tumors with a Ki-67 LI of < 20%, but 5-8-fold higher in the remaining 4 cases. In contrast, the kinase activities in all 8 HCC cases in a subgroup with a Ki-67 LI of > 20% were significantly higher (20-fold-50-fold higher than their matched non-tumorous liver samples). Heating the tumor sample lysates effectively reduced the CDK2 activities, indicating that p27 *per se* was functional and not inactivated by either DNA mutations or genetic loss, since p27 is heat-stable. These lines of evidence indicate that p27 does not simply act as a tumor suppressor in some types of cancer cells, and that a thorough examination of the biological status of p27 should be performed.

FUNCTIONAL INACTIVATION OF p27 IS CLOSELY ASSOCIATED WITH SEQUESTRATION TO CYCLIN D1-CDK4-COMPLEXES

To understand the molecular mechanism of the different levels of kinase activities among the high-p27 expressers, examination of the components of p27-containing complexes may be useful, since it has been reported that sequestration of p27 from CDK2 to cyclin D1-containing complexes results in a blockade of p27 functions as well as stabilization of active cyclin D1-CDK4/CDK6 complexes^[51,52]. Therefore, we performed immunoprecipitation assays using 20 tissue sample lysates from high-p27 expressers and found that, in 12 tumors with low levels of kinase activities (< 8-fold relative to the levels in adjacent non-tumorous tissues), p27 was closely

associated with CDK2 in 8 samples and faintly associated with cyclin D1-CDK4 complexes in 4 samples. In contrast, in all 8 HCC samples with high levels of kinase activities (20-fold-50-fold higher relative to the levels in adjacent non-tumorous tissues), high levels of cyclin D1-CDK4-bound p27 were detected. These pieces of evidence strongly indicate that compositional changes in the complexes containing p27 may be an alternative reason for the increased cell proliferation in p27-overexpressing HCC.

THE COMPOSITIONS OF COMPLEXES CONTAINING p27 VARY AMONG HIGH p27 EXPRESSERS

Several cell-cycle regulatory factors are known to influence the compositional status of p27-containing complexes. For example, cyclin D1 and CDK4 mobilize p27 from cyclin E-CDK2 complexes to cyclin D-CDK4 complexes, while p15 and p16 shift p27 to associate with cyclin E-CDK2 complexes^[53,54]. We examined the expressions of these cell-cycle regulatory factors in high-p27 expressers, and found that p16 was closely correlated with the status of complexes containing p27, while CDK4 and cyclins D1, D3 and E were not. Our results revealed that p16 was expressed in 9 of 12 high-p27 expressers in which p27 was predominantly associated with CDK2, but was undetectable in all 8 HCCs in which p27 was closely associated with cyclin D1-CDK4 complexes^[6]. Although there have been no other reports of the molecular relationships between p16 and p27-containing complexes, it is noteworthy that Sanchez-Beato *et al* reported a close relationship between p16 loss and anomalous p27 expression in aggressive B-cell lymphomas^[55]. Further studies are needed to address whether the status of p16 influences any clinicopathological characteristics in human tumors expressing high levels of p27.

p16 DETERMINES THE PROGNOSIS OF HCCS WITH p27 OVEREXPRESSION

To further investigate the functional role of p16 in the cell proliferation status of high-p27 expressers, we examined the LIs of p16 and Ki-67 in HCCs and found that the p16 LI was inversely correlated with the Ki-67 LI in high-p27 expressers. Immunohistochemical analyses revealed that p16 staining was positive in all 26 (100%) high-p27 expressers with a Ki-67 LI of < 20%, but negative in 12 of 14 (85%) high-p27 expressers with a Ki-67 LI of > 20%. Interestingly, methylation of the *p16* gene promoter was not detected in all 26 (100%) samples with positive p16 immunostaining, but was detected in 10 of 12 (83%) samples with negative p16 immunostaining. Therefore, we surmise that epigenetic changes of the *p16* gene may be the main cause of the p27 inactivation in HCCs that express considerable amounts of p27. Since we also found that loss of p16 expression was an independent prognostic factor for a poor outcome in high-p27 expressers compared with standard prognostic variables^[6],

assessment of the *p16* status may be useful for precise prognostic prediction in individuals with HCCs expressing high levels of p27.

FORKHEAD TRANSCRIPTION FACTORS AND p27

Recently, Forkhead box M1B (Foxm1b), a ubiquitously expressed member of the Forkhead Box (Fox) transcription factor family, has been focused upon as another regulatory factor of p27 expression. Foxm1b expression is restricted to proliferating cells and mediates both hepatocyte entry into DNA synthesis and mitosis during liver regeneration^[56,57]. Wang *et al* showed that regenerating livers in aged mice exhibited diminished Foxm1b expression with significant reductions in hepatocyte proliferation and increased levels of p27 expression^[58], thereby providing new evidence that Foxm1a regulates p27 at the protein level. Kalinichenko *et al* reported that Foxm1b was essential for the development of a rodent HCC model, and its mechanism was associated with reduced expression of p27 and increased expression of the CDK1-activator Cdc25B phosphatase^[59]. More significantly, they also showed that Foxm1b was a novel inhibitory target of the p19 (ARF) tumor suppressor, and that a peptide containing p16 may represent a potential therapeutic agent toward Foxm1b overexpressing cancer cells. Since FoxM1 is essential for transcription of Skp2 and Cks1^[60], which are specificity subunits of the SCF ubiquitin ligase complex that targets p27, examining the relationship between Foxm1b and p27 in human HCC may help toward understanding of the molecular mechanism of hepatocarcinogenesis.

CONCLUSION

HCC is one of the most common human cancers, and its incidence is still increasing in civilized countries. HCC represents the fifth most-common malignant disease in the world and is the third most-common cause of cancer-related death worldwide^[61,62]. The etiology of HCC is unique in that most HCC cases are associated with liver cirrhosis or chronic hepatitis attributable to HBV or HCV infection, chronic alcohol abuse and, more recently, non-alcoholic steatohepatitis^[63]. Since the prognosis of HCC patients remains poor, identification of useful molecular prognostic markers for HCC is required. As described in the present review, coordinated examination of the p16 and p27 statuses may become a more accurate tool for predicting the prognosis of HCC. Further studies of cell-cycle regulators will provide better insights into the mechanism of hepatocarcinogenesis.

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DNA methylation in hepatocellular carcinoma

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Abstract

As for many other tumors, development of hepatocellular carcinoma (HCC) must be understood as a multistep process with accumulation of genetic and epigenetic alterations in regulatory genes, leading to activation of oncogenes and inactivation or loss of tumor suppressor genes (TSG). In the last decades, in addition to genetic alterations, epigenetic inactivation of (tumor suppressor) genes by promoter hypermethylation has been recognized as an important and alternative mechanism in tumorigenesis. In HCC, aberrant methylation of promoter sequences occurs not only in advanced tumors, it has been also observed in premalignant conditions just as chronic viral hepatitis B or C and cirrhotic liver. This review discusses the epigenetic alterations in hepatocellular carcinoma focusing DNA methylation.

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Key words: Hepatocellular carcinoma; DNA methylation; Histone modification; Tumor suppressor genes

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INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common cancer worldwide. It shows a wide geographical variation with low incidence areas in North America and Europe and high incidence areas in Africa and Asia.

70%-80% of hepatocellular carcinoma occurs in cirrhotic liver. In high incidence areas, such as Asia and Africa, HCC is strongly associated with chronic viral hepatitis B and C and liver cirrhosis. Nutritional factors, toxins and metabolic diseases contribute also to hepatocarcinogenesis^[1,2].

As for many other tumors, development of HCC is due to a multistep process with accumulation of genetic and epigenetic alterations in regulatory genes, leading to activation of oncogenes and inactivation or loss of tumor suppressor genes (TSG).

In the last three decades, cancer has been understood as a summary of altered genetic and epigenetic events. The epigenetic pathway is, in contrast to genetic events, a reversible alteration and characterized by three main mechanisms: (1) DNA hypermethylation leading to inactivation, (2) DNA hypomethylation causing genomic instability, (3) histone modifications affecting chromatin conformation.

These processes, especially aberrant DNA methylation and histone modifications, are closely linked with each other by a protein complex of transcript activators and repressors and alter mRNA transcript expression of affected genes^[3].

Characteristically, DNA methylation does not change the genetic information. It just alters the readability of the DNA and results in inactivation of genes by subsequent mRNA transcript repression.

In humans and other mammals, CpG island methylation is an important physiological mechanism. The inactivated X-chromosome of females, silenced alleles of imprinted genes or inserted viral genes and repeat elements are inactivated through promoter methylation^[4,5].

DNA HYPERMETHYLATION

Promoter hypermethylation

Hypermethylation of CpG islands in promoter sequences is associated with silencing of tumor suppressor genes and tumor-related genes by subsequent downregulation of mRNA transcript expression. Epigenetic silenced genes are involved in important molecular pathways of carcinogenesis e.g., cell cycle regulation, apoptosis, DNA repair or cell adhesion.

According to other types of malignant tumors, in hepatocellular carcinomas, aberrant methylation of several TSG and tumor-related genes such as *RASSF1A*, *bMLH1* or *SOC31* was frequently observed^[6].

CpG island hypermethylation is not only seen in HCC

Table 1 Methylation in hepatocellular carcinoma

Gene	Location	Function	Methylation frequency (%)	Ref.
p16 ^{INK4a}	9q21	CDK inhibitor	17-83	[10,11]
p14 ^{ARF}	9q21	MDM2 inhibitor	25-30	[7,12]
CASP8	2q33	Apoptosis	72	[17]
TMS1/ASC	16p11.2	Apoptosis	80	[23]
E-Cadherin	16q22.1	Cell adhesion	33-67	[28-30]
M-Cadherin	16q24.1	Cell adhesion	55	[34]
H-Cadherin	16q24.2-3	Cell adhesion	21	[17]
TIMP3	22q12	MMP inhibitor	13-19	[37-39]
hMLH1	3p21.3	Mismatch repair	18-44	[47-50]
hMSH2	2p21-22	Mismatch repair	68	[47,49,50]
hMSH3	5q11-12	Mismatch repair	75	[47,49,50]
MGMT	10q26	DNA repair	22-39	[53-55]
GSTP1	11q13	Detoxification	41-58	[53,58-61]
SOCS-1	16p13.13	Cytokine inhibitor	60	[67]
SOCS-3	17q25.3	Cytokine inhibitor	33	[68]
RASSF1A	3p21.3	Apoptosis	54-95	[71,75,76]
BLU	3p21.3	Unknown	20	[71]
SEMA3B	3p21.3	Apoptosis	80	[71]
FHIT	3p14.2	histidine triad protein	71	[86]

tumor tissue. Even in premalignant conditions such as dysplastic nodules or cirrhotic liver, promoter methylation of several kinds of TSG, e.g. E-cadherin, *GSTP1* or *p16^{INK4a}* was demonstrated (Table 1 shows promoter methylation of different tumor-related genes in HCC).

Proliferation and apoptosis

One of the most important pathways affected in HCC are the Rb (Retinoblastoma) gene and *INK4a*-ARF pathway^[7]. The *INK4a*-ARF locus is coding two cell-cycle regulatory proteins, *p16^{INK4a}* and *p14^{ARF}*, acting through the Rb-CDK4 and p53 pathways. *p16^{INK4a}* binds to cyclin-dependent protein kinase 4 (CDK4) and inhibits the ability of CDK4 to interact with cyclin D1. *p14^{ARF}* prevents the p53 degradation through its binding to MDM-2 and induces cell cycle arrest^[8,9].

p16^{INK4a} is one of the most altered tumor suppressor gene in human cancer. In HCC, loss of *p16^{INK4a}* is mainly caused by aberrant promoter methylation, whereas deletions and mutations of this gene locus are infrequently seen. CpG island promoter methylation was reported from 55% to 73%, but aberrant methylation occurred also in non-cancerous liver tissue with cirrhosis in 29% or chronic hepatitis B and C up to 23%^[10,11]. Compared to *p16^{INK4a}* methylation, *p14^{ARF}* promoter methylation was observed less frequently in 8% to 20% of HCC. It was demonstrated that inactivation of *p14^{ARF}* is due to homozygous deletions. No correlation was found between p53 mutations and promoter methylation of *p16^{INK4a}* or *p14^{ARF}*^[7,12].

Caspase 8 (CASP8) is a key apoptotic gene that is involved in death receptor and the mitochondrial pathways and acts as initiator CASP^[13]. *CASP8* is silenced by aberrant hypermethylation of its promoter in childhood neuroblastomas^[14,15]. In HCC, *CASP8* aberrant promoter methylation was reported by Yu *et al*^[16] with a frequency up to 72%.

TMS1/ASC, another proapoptotic gene, functions as a negative regulator of nuclear factor kappaB (NF-κB) and

blocks transcription of survival signals^[17]. First, *TMS1* was identified as a target of methylation-induced silencing in cell lines overexpressing DNMT1. Epigenetic inactivation of *TMS1* was demonstrated in human glioblastomas, ovarian cancer, human melanoma, colorectal carcinomas or in lung cancer and breast cancer^[18-22]. In HCC, *TMS1* promoter methylation was observed in 80%^[23].

In hepatocellular carcinoma cell lines, restoration of *TMS1* transcript was induced by demethylating agent 5'-AZA and trichostatin, a histone deacetylase inhibitor. Furthermore, in these cell lines *TMS1* DNA methylation was associated with histone H3 lysine 9 hypoacetylation and trimethylation^[24].

Cell adhesion and invasion

E-cadherin (CDH1): E-cadherin, a member of calcium-mediated membrane glycoproteins, is expressed in all epithelial cells acting as an adhesion molecule. Inactivation of E-cadherin induces loss of adherens junctions and impairment of cell adhesiveness and cell proliferation signalling pathways. In tumours, reduction of E-cadherin expression results in tumour progression, cell invasion and formation of metastasis^[25,26].

Downregulation of E-cadherin, caused by genetic and epigenetic mechanism, is a frequent event in most type of epithelial carcinomas. In poorly-differentiated breast and gastric cancer, somatic mutations of E-cadherin are frequently found. Further, in all cases of familial gastric cancer, loss of E-cadherin is mainly caused by germline mutations^[27]. In other types of tumors, mutations are infrequent events and repression of E-cadherin is mainly caused by aberrant promoter methylation.

In according to CC, mutations of E-cadherin are rare events in HCC. Reduced or loss of E-cadherin expression is mainly caused by aberrant CpG island methylation with a detectable frequency from 33% to 67%^[28-30]. Wei *et al*^[31] described, that loss of E-cadherin was closely associated with loss of heterozygosity (LOH) of E-cadherin and CpG hypermethylation. In precancerous conditions just as dysplastic nodules or liver tissue with chronic hepatitis or cirrhosis, aberrant E-cadherin methylation was detected in 8% and 46%, respectively^[29,30].

Other factors, except epigenetic inactivation or mutations, leading to inactivation of E-cadherin include transcriptional repression by binding of transcriptional factors, e.g. the repressors Snail or Sip-1 to CDH1-E box elements^[32,33].

But not only E-cadherin, as a member of cadherin genes, is epigenetically altered in HCC. Yamada *et al*^[34] reported the highest methylation frequency of M-cadherin with a frequency to 55% among seven elucidated cadherin genes. Methylation-induced silencing of H-Cadherin (CDH13) was observed by Yu *et al*^[17], reaching 21%.

TIMP-3: Tissue inhibitor of metalloproteinase-3 (*TIMP-3*) leads to inhibition of cell migration and angiogenesis. In human carcinoma cell lines, overexpression of *TIMP-3* suppresses cell growth and induces apoptosis by stabilization of TNF-alpha receptors on the cell surface^[35,36]. A *TIMP-3* downregulation was demonstrated

in different kinds of tumors, mostly mediated by CpG island promoter methylation^[6].

In HCC, *TIMP-3* methylation is an infrequent event reaching 13% to 19%. No methylation was found in normal liver tissue. Lü *et al.*^[37] demonstrated *TIMP-3* methylation in 25% of hepatocellular cancer emboli in portal veins. The aberrant promoter methylation is accompanied by loss or reduced *TIMP-3* mRNA and protein expression^[37-39].

TFPI-2: *TFPI-2* is a Kunitz-type serine protease inhibitor that represses cellular invasion in several kinds of tumors, e.g. in lung cancer or pancreas carcinomas, by suppressing plasmin-mediated activation of MMP-1 and MMP-3 or inhibition of plasmin and trypsin activity^[40-43].

Wong *et al.*^[44] observed *TFPI-2* downregulation with reduced or loss mRNA transcript expression in HCC with a frequency of 90%. In 47% of the observed HCC, aberrant CpG methylation was seen, but not in normal liver tissue. In HCC cell lines with epigenetically induced silencing, a *TFPI-2* mRNA transcript re-expression was induced by combined treatment with the demethylating agent 5'-AZA-DC and the histone deacetylase inhibitor TSA.

DNA repair

Mismatch repair system: Defects in DNA repair mechanisms may result in accumulation of mutations and genomic instability. The mismatch repair system (MMR) is one of the most important DNA repair mechanisms correcting errors in DNA replication. Defects of the MMR leading to microsatellite instabilities (MSI) have been observed in approximately 15% of sporadic colorectal and gastric carcinomas^[45,46]. Promoter methylation of MMR genes in HCC occurred with a frequency of 5% to 13% for *bMLH1*, 68% for *bMSH2* and 75% for *bMSH3*. A high methylation frequency of *bMSH2* and *bMSH3* was observed in HCC corresponding non neoplastic liver tissue, especially in cirrhotic liver tissue, reaching 55 % and 70%, but not in normal liver tissue. No correlation was found neither to viral hepatitis nor to MSI status and DNA methylation of analyzed MMR genes^[47-50].

MGMT (O6-methylguanine DNA methyltransferase): O6-methylguanine DNA methyltransferase (*MGMT*) is another important DNA repair gene with the highest activity in the liver^[51]. *MGMT* protects cells from DNA damage caused by mutagenic and cytotoxic agents leading to alkylation at O6-guanine. Loss or reduced *MGMT* expression due to CpG islands methylation was detected in several kinds of human cancers^[52]. In HCC, aberrant methylation occurred with a frequency of 22% to 39%, whereas the *MGMT* promoter shows higher methylation levels in chronic viral hepatitis associated HCC^[53-55]. Interestingly, Su *et al.*^[53] reported that *MGMT* promoter methylation occurred to a similar extent in non neoplastic liver tissue compared to HCC.

GSTP1 (Glutathione S-transferase P1): The detoxifying glutathione S-transferase P1 (*GSTP1*) gene protects cells from cytotoxic and carcinogenic influences

in due to inactivation of electrophilic carcinogens by conjugation with glutathione. Promoter methylation of *GSTP1* is best analyzed in prostate cancer. *GSTP1* methylation is an early event in prostatic carcinogenesis, because in high-grade prostatic intraepithelial neoplasia loss of *GSTP1* expression is caused by DNA methylation. Many other tumor types including breast cancer and cholangiocarcinoma showed a *GSTP1* hypermethylated promoter^[56,57]. In HCC, methylation of the *GSTP1* gene occurred in 41% to 85%^[53,58-61]. Zhang elucidated *GSTP1* methylation in HCC in presence of environmental chemical carcinogens. A significant correlation was observed with higher aflatoxin B₁ (AFB₁)-DNA adducts in tumor tissue in contrast to tumor tissue without or lower levels of AFB₁-DNA adducts. However, no association was found between *GSTP1* methylation and polycyclic aromatic hydrocarbon-DNA adducts^[59]. So far, aberrant methylation of *GSTP1* is not only detectable in tumor tissue, Wang *et al.*^[62] observed a *GSTP1* hypermethylation in serum of HCC patients.

Suppressors of cytokine signaling (SOCS): Suppressors of cytokine signaling 1 and 3 (SOCS-1 and SOCS-3) are intracellular proteins that act as negative regulators of Janus kinase (JAK) and signal transducer and activators of signaling pathways (STAT). The JAK/STAT signaling pathway plays an important role in cell growth and differentiation or immune reaction mediated by cytokines. Cytokines activate JAK's by binding to membrane receptors that leads to phosphorylation of STAT's and activates target genes. *SOCS1* and *SOCS-3* bind direct and indirect to JAK's and inhibit the phosphorylation of STAT's and activation of target genes^[63,64].

Aberrant methylation of *SOCS-1* and *SOCS-3* promoter sequence has been reported in several kinds of human cancer. *SOCS-1* and *SOCS-3* CpG island hypermethylation is an early event in human carcinogenesis. Recently, we have shown methylation-induced downregulation of *SOCS-1* and *SOCS-3* in precursor lesions of Barrett's adenocarcinomas and precursor lesions of squamous carcinomas of head and neck^[65,66]. In HCC, aberrant promoter methylation of *SOCS-1* and *SOCS-3* occurred with a frequency of 60% and 33%, respectively^[67,68]. Methylation of the *SOCS-1* gene was detected in HCV-induced chronic hepatitis and liver cirrhosis, reaching 45%, whereas the methylation frequency increased with fibrosis stage with the highest proportion in liver cirrhosis^[69,70].

Methylation hot spot 3p

The short arm of human chromosome 3 belongs to regional methylation hot spots in addition to chromosomal locus 11p and 17p. Alterations of the genetic information on chromosome 3 are one of the most frequent and earliest steps in the carcinogenesis of several types of tumors. LOH of chromosome 3p occurred in about 30% of hepatocellular carcinomas^[71].

In different kinds of human cancer, epigenetic inactivation *via* promoter methylation of several genes located on 3p, including *RASSF1A* on 3p21.3, *bMLH1* at 3p21.3, *RARB* 2 at 3p24.2, was shown.

One of the most frequent observed and most

epigenetically inactivated genes of 3p is *RASSF1A*, a multifunctional tumor suppressor gene that protects cells from genomic instability and transformation by stabilizing the microtubules^[72,73]. An aberrant promoter methylation was detected in about 50% of malignant tumors. In renal cell carcinoma and small cell lung cancer, the highest prevalence was observed, reaching about 91%^[74]. In HCC, hypermethylation occurred in approximately 54% to 95%, whereas HBV-associated HCC showed higher levels of *RASSF1A* methylation compared to HCC without risk factors. *RASSF1A* methylation occurs not only in HCC, methylation is even observed in non-neoplastic precancerous conditions like cirrhotic liver and chronic hepatitis^[71].

Semaphorin 3B (*SEMA3B*) and *BLU* are two other putative tumour suppressor genes located on 3p21.3, whereas the function of *BLU* still remains unclear. In lung cancer, *BLU* overexpression inhibits tumor colony formation efficiency. Qiu *et al*^[77] reported that *BLU* might function as an environmental stress-responsive gene, regulated by E2F, at least in nasopharyngeal carcinomas. However, *BLU* methylation is a rare event in human cancer. We detected *BLU* promoter methylation in about 20% of our examined HCC^[71,78].

SEMA3B, a member of the Semaphorin family, suppresses tumor formation in lung cancer and induces apoptosis. It has been demonstrated that *SEMA3B* induced apoptosis is antagonized by *VEGF*¹⁶⁵ in due to an interaction with NP-1 receptor^[79,80].

Aberrant methylation of *SEMA3B* was detected in lung cancer and gliomas^[81,82]. We reported a high prevalence of *SEMA3B* methylation in HCC, reaching 80%. In contrast, the tumor surrounding non-neoplastic liver exhibited an unmethylated *SEMA3B* promoter. Further, *RASSF1A* and *SEMA3B* expression was restored by treatment with the demethylating drug 5-AZA-C in HCC cell lines, suggesting that promoter hypermethylation is responsible for silencing transcript expression^[71,72,81].

The fragile histidine triad (*FHIT*) gene, located to 3p14.2, embraces FRA3b, the most actively fragile site in humans^[83,84]. Functional and structural alterations of *FHIT* were identified in several kinds of human cancer. Methylation induced silencing was described in lung and breast cancers^[85]. In HCC, promoter methylation of *FHIT* is a frequent and early event. Sun *et al*^[86] observed *FHIT* hypermethylation with a frequency of 71% in HCC, 64% in non neoplastic liver tissue and 14 % in normal liver.

CpG island methylator phenotype (CIMP)

Carcinomas with high rates of accumulated aberrant promoter methylation of tumor-related genes are characterized as CIMP⁺ (CpG island methylator phenotype). CIMP⁺ was first described for colorectal and gastric cancer by Toyota and Issa in 1999^[87,88]. Shen *et al*^[89] reported that CIMP⁺ is associated with environmental exposures in HCC. HCC from patients without precancerous conditions or risk factors, respectively, showed significantly lower levels of methylation than HCC arising from patients with chronic hepatitis B and C or patients with cirrhosis.

CIMP positive HCC (tumours with five genes that

are concordantly methylated) showed a significantly association with methylation of the TSG *p14*, *p15*, *p16* ER, *RASSF1A* or *WT1* and elevated serum alpha-fetoprotein (AFP) levels. Further, CIMP⁺ was commonly seen in HCC with increased serum AFP levels^[90,91].

DNA-methyltransferases (DNMT)

DNA hypermethylation is catalyzed by the family of DNA methyltransferases (DNMT) including DNMT1, DNMT3a and DNMT3b. DNMT1 is required for maintenance of DNA methylation whereas DNMT3a and DNMT3b function as de novo DNA methyltransferases^[92-94]. DNMT2 was former described as DNMT because of its strong similarity with m5C methyltransferases of pro- and eukaryotes. But it was recently shown that DNMT2 does not methylate DNA. It's the first described RNA cytosine methyltransferase that methylate position 38 in Aspartic acid transfer RNA^[95].

In human cancer just as in HCC, an upregulation of DNMT activity is seen in contrast to global hypomethylation. Park *et al*^[96] described a significantly overexpression of DNMT1 and DNMT3b in HCC compared to non-neoplastic liver tissue. DNMT3a showed similar or higher expression levels. Saito *et al*^[97] observed higher expression levels of all three DNMTs in HCC and cirrhotic liver than in normal liver. Increased DNMT1 and DNMT3a expression was also reported in dysplastic nodules^[98].

According to other tumors, no correlation was seen between DNMT upregulation and promoter hypermethylation-induced inactivation of tumor-related genes. The certain mechanisms of DNMT upregulation remains still unclear, but it is suggested that aberrant DNMT activity, especially of DNMT1, is due to rapid proliferation of cancer cells because DNMT1 binds to proliferating cell nuclear antigen (PCNA)^[99-101].

DNA hypomethylation on pericentromeric satellite regions results in chromosomal instability. During hepatocarcinogenesis, DNA hypomethylation of these regions was reported in HCC and precancerous conditions. The splice variant of DNMT3b, DNMT3b4 that may lack DNA methyltransferase activity is associated with DNA hypomethylation on pericentromeric satellite regions. Saito *et al*^[102] reported that overexpression of DNMT3b4 was seen in cirrhotic liver, chronic hepatitis and HCC whereas increased DNMT3b4 levels correlated with DNA hypomethylation on pericentromeric satellite regions.

DNA HYPOMETHYLATION

In human cancer, global DNA hypomethylation leads to genomic instability, affects repeated DNA sequences, tissue-specific genes and proto-oncogenes or causes loss of imprinting with a biallelic expression, just as in case of *IGF2*. Further, the level of DNA hypomethylation increases with tumor progression^[103,104]. In recent years, DNA hypomethylation was shown in several human cancer and some premalignant alterations, i.e. colorectal adenomas and carcinomas, adenocarcinoma of prostate, breast cancer or intestinal type of gastric carcinoma and hepatocellular carcinoma, respectively^[105-108].

Lin *et al.*^[109] observed 5-methylcytosine (m5C) content in hepatocellular carcinogenesis by comparing hepatocellular carcinoma with non neoplastic liver, including cirrhotic livers. In all cancer tissues, 5-methylcytosine was significantly reduced. No difference of the m5C content was detected in cirrhotic and non cirrhotic liver tissue. The reduced 5mC level was associated with large tumor size and poorly histopathological grade.

It is suggested that (re-)activation of retroposons might be associated with global hypomethylation because approximately 90% of all m5C lies in these elements. An association between hypomethylation and transposon activation, especially of LINE-1 transposons, has been observed in human testicular carcinoma cell lines, urothelial carcinoma cell lines and teratocarcinoma cell lines^[110-112]. But in HCC, an activation of transposable elements just as LINE-1 retrotransposons *via* hypomethylation could not be detected yet^[109].

HISTONE MODIFICATION

Histone modifications are strongly associated with formation of the nucleosome structure and are closely linked to CpG island methylation by interacting with Methyl-CpG-binding proteins (MBD's) and DNA methyltransferases (DNMT's). Modifications including methylation, acetylation or phosphorylation of certain position of the histone tails. Whereas histone methylation is associated either to activation or to repression, histone hypoacetylation mediated by histone deacetylases leads mostly to DNA relaxation and subsequent accessibility for transcriptional factors with repression of transcription.

Lee *et al.*^[113] reported that HCC with low survival expressed higher levels of genes involved in histone modifications just as PTMA and SET, two proteins that are members of inhibitors of histoneacetyltransferases complex.

P73, a member of the TP53 family represses AFP expression during normal hepatic development by chromatin structure alterations. In hepatoma cells, transactivated p73 suppresses endogenous AFP transcription via reducing of acetylated histone H3 lysine 9 and increasing dimethylated histone H3 lysine 9^[114].

CONCLUSION

In hepatocarcinogenesis, aberrant methylation of tumor related genes occurs not only in advanced tumour stages, it's a frequent and early event. Promoter methylation of different kinds of tumor suppressor genes including *p16*, *SOC31* and *SOC33* or *RASSF1A*, has been demonstrated in premalignant conditions just as chronic hepatitis or liver cirrhosis. Moreover, the frequency of aberrant promoter methylation increases during the progression from precancerous lesion to HCC. In HBV or HCV-associated chronic hepatitis, methylation frequency of detected genes is significantly higher than in non-neoplastic non-viral liver tissue. Therefore, epigenetic changes in preneoplastic or early neoplastic stages may serve as indicator or "biomarker" for screening of patients with an increased risk for HCC.

Further, HCC is one of the most common causes of cancer death worldwide with a poor prognosis. Only few therapeutic interventions exist. It was demonstrated that re-expression of tumor suppressor genes that are epigenetically silenced is possible by using demethylating and histone modifying agents. In the next years, this might be a possible therapeutic approach analogue to other malignant diseases, e.g. myelodysplastic syndrome, but the used therapeutic agents that influence DNA hypermethylation are toxic and lead to genome wide alteration of the methylation pattern with possible activating of oncogenes or imprinted genes. Another possible aspect of chemotherapy might be to modulate the epigenetically involved pathways by using small molecules that are more specific. But further investigations in clinical trials are needed to prove and integrate epigenetic pathway modulating agents.

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Genome-wide differences in hepatitis C- vs alcoholism-associated hepatocellular carcinoma

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Abstract

AIM: To look at a comprehensive picture of etiology-dependent gene abnormalities in hepatocellular carcinoma in Western Europe.

METHODS: With a liver-oriented microarray, transcript levels were compared in nodules and cirrhosis from a training set of patients with hepatocellular carcinoma (alcoholism, 12; hepatitis C, 10) and 5 controls. Loose or tight selection of informative transcripts with an abnormal abundance was statistically valid and the tightly selected transcripts were next quantified by qRT-PCR in the nodules from our training set (12 + 10) and a test set (6 + 7).

RESULTS: A selection of 475 transcripts pointed to significant gene over-representation on chromosome 8 (alcoholism) or -2 (hepatitis C) and ontology indicated a predominant inflammatory response (alcoholism) or changes in cell cycle regulation, transcription factors and interferon responsiveness (hepatitis C). A stringent selection of 23 transcripts whose differences between

etiologies were significant in nodules but not in cirrhotic tissue indicated that the above dysregulations take place in tumor but not in the surrounding cirrhosis. These 23 transcripts separated our test set according to etiologies. The inflammation-associated transcripts pointed to limited alterations of free iron metabolism in alcoholic vs hepatitis C tumors.

CONCLUSION: Etiology-specific abnormalities (chromosome preference; differences in transcriptomes and related functions) have been identified in hepatocellular carcinoma driven by alcoholism or hepatitis C. This may open novel avenues for differential therapies in this disease.

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Key words: Alcoholism; Chromosome; Cirrhosis; Hepatitis C; Transcriptomes; Protein function

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INTRODUCTION

Hepatocellular carcinoma (HCC) is a primary liver cancer, the main causes of which are viral hepatitis (HBV; HCV), alcoholism or aflatoxin B1 intoxication. In most instances HCC develops in the setting of chronic hepatitis and/or cirrhosis. Numerous HCC-associated genomic and/or epigenomic alterations result in a dysregulated expression of genes and proteins^[1,2]. Liver transcriptome analysis by microarray has resulted in the identification of numerous genes with an aberrant expression in HCC as compared to the surrounding cirrhosis^[2-6]. Although mRNA down-regulation predominates in this context^[7], HCC-associated gene expression profiles largely vary between patient

subgroups^[6,8]. This feature is of prognostic interest as different profiles are associated with the evolution rate or the occurrence of metastasis and relapse^[2-4,9-11]. However, a comprehensive picture of altered gene regulation in HCC remains elusive^[2,5,12]. Notably, the variety of etiologies with their associated abnormalities at the genome level is likely to promote distinct gene dysregulations and hence creates further complexity. For instance, the HBV- or HCV-induced genetic alterations are known to be different^[9] and the associated transcriptomes have proven to vary significantly^[13,14]. Therefore, deciphering the transcriptome patterns as a function of HCC etiology is of critical importance. However, the gene dysregulations in the context of alcohol abuse are poorly understood^[15] and the associated transcriptome has seldom been studied. *A fortiori*, a comparison of liver transcriptomes in HCV virus- vs alcoholism-associated HCC has never been done. We now report that, among various findings, these transcriptomes differ in the cancerous nodules whereas, unexpectedly, they remain similar in the surrounding cirrhosis. This points to etiology-dependent mechanisms that take place at a relatively late stage of tumoral transformation.

MATERIALS AND METHODS

Human subjects and RNA sources

Liver fragments were obtained under strict anonymity from the digestive surgery unit of Charles Nicolle Hospital (Rouen, France). A fragment of a cancerous nodule as well as distant cirrhotic tissue were taken whenever an HCC resection was performed. In multinodular livers, only 1 nodule was studied, provided the tumor grade of this nodule was known. Control human livers were non-tumorous tissue from patients operated for benign liver tumor. Histopathology was carried out by a trained pathologist (AF). According to the current French rules and ethical guidelines, neither an informed consent nor advice from an ethical committee are requested prior to analysis of RNA in resected tissues that would otherwise be disposed off. Tissue storage and RNA extraction were done as described^[16].

HCV- vs HBV-infection vs chronic alcohol abuse

Chronic alcohol abuse was defined as a regular, daily consumption of > 80 g or > 60 g ethanol in men or women, respectively, as estimated from three cumulated criteria, namely: (1) alcohol consumption, as indicated by the patient, (2) alcohol dependency, as evaluated from a specific interview and (3) blood level of several hepatic proteins (alanine aminotransferase, aspartate aminotransferase, gammaglutamyl transpeptidase). HBV infection was serologically assessed with the HBs antigen and anti-HBc antibodies (AxSYM kits from Abbott Laboratories). HCV infection was serologically determined by enzyme immunoassay (AxSYM HCV-3.0 kit from Abbott Laboratories). Both infections were further searched at the nucleic acid level in all patients of this study. HBV DNA was detected as described^[17] in genomic DNA extracted from paraffin-embedded liver samples (DEXPAT Kit from TaKaRa Laboratories). HCV RNA

was detected in 2 µg hepatic RNAs with the Abbott real-time HCV kit (Abbott Laboratories, France). Serological and genomic determinations were consistent in all cases.

Transcriptome analysis and real-time qRT-PCR

Our set of human cDNA probes dubbed *Liverpool* that is tailored to a complete coverage of the human transcriptome in healthy or cancerous liver (*ca.* 10⁴ genes), the associated *LiverTools* database, as well as the procedures from array preparation to final data handling have all been detailed^[16]. In brief, every RNA sample was subjected to 3 hybridization replicates. The resulting, normalized values were used for selection of significantly up- or down-regulated mRNA in cirrhosis vs paired nodule, using a statistically validated, funnel-shaped confidence interval ($P < 0.05$) calculated from every mRNA detected per hybridization. This resulted in a false discovery rate (FDR) that is below 10% of the total number of regulated mRNAs, in agreement with an FDR estimate obtained from other, cumulated analyses in liver^[5]. Abnormal mRNA ratio in cirrhosis vs paired nodule was defined from a statistically different abundance in at least 2 out of 3 replicates. A control of every cDNA probe was done by DNA re-sequencing with an ABI3100 capillary sequencer (Applied Biosystems). Real-time qRT-PCR of mRNAs was done as described^[16] with primers designed with the Primer3 software (<http://frodo.wi.mit.edu>), and normalized with the 18S RNA level. The primers were: *AGXT*, forward CGCTGGCTATGACTGGAGAG, reverse GTCACGCGGTCCACATTCT, amplicon size 150 bp; *APCS*, TGGGAGAGATTGGGGATTG, CCACACCAAGGGTTTGATGA, 158 bp; *APOC3*, ACTGAGCAGCGTGCAGGAG, TCACGGCTGAAGTTGTCTGA, 154 bp; *ATP6-V0D1*, TACCTCAACCTGGTGCAGTG, GTCTAGGAAGCTGGCGAGTG, 198 bp; *C4A*, TTGATC-ATGGGTCTGGATGG, CCTGGAGGA-AGTCGT-TGAGC, 157 bp; *CES1*, GGGTGCCTCAGAAG-AGGAGA, CTGGGTGTTGGCACCAATCT, 154 bp; *CLU*, ATGTTCCAGCCCTTCCTTGA, TCGTCGCCT-TCTCGTATGAA, 112 bp; *COBL*, TGGCATCCTCTGCTTCTGAG, CGTCCTTGGTGCAGAGAGAG, 161 bp; *CRP*, TCGTATGCCACCAAGAGACA, CTTCTGCCCCCACAGTGAT, 235 bp; *CYP2E1*, TCAAGCCATTTTCACAGGA, CGATATCCTTTGGGTCAACGA, 129 bp; *FGL1*, GAAATTCAG-CACGTGGGACA, CCATTGTCTGTTTAGCCGTGT, 150 bp; *HP*, AACTGCGCACAGAAGGAGAT, TGGTGGGAAACCATCTTAGC, 202 bp; *HPR*, AGGGCGTGTGGGTTATGTTT, TTCTTTTCGGGGACTGTGCT, 141 bp; *HPX*, TGTGGATGCGGCCCTTTATCT, GGCCAAGGGACTTTTCCATA, 167 bp; *IDH2*, AAAGATGGCAGT-GGTGTCAAG, TCATGTACAGCGGCCATTTC, 151 bp; *MAGI1*, GGCAATGCATGTGTGGCTAT, CATCCATTTACTGCCAAGATCC, 113 bp; *NNMT*, CCCTCGGGATTACCTAGAAAAA, AGAGCCGATGTCAATCAGCA, 145 bp; *PDI43*, AGAACTCACGGACGACA ACTTC, GCAGTGCAATCAACCTTTGC, 177 bp; *PSMD10*, GCATCCACAACATCCAAGA, TACTTGCTCCTTGGGACACC, 106 bp; *RBP4*, GATGGCACCTGTGCTGACA, TCGCAGTAACCGTTGTGGAC, 149 bp; *S4A*,

TTTTCTGCTCCTTGGTCCTG, GAATGAGG GGTGCTCTTTCA, 161 bp; *SCARB2*, TTTGATCA TCACCAACATACCC, ATCATAGTTCCCCCG-AGCAT, 134 bp; *18S*, GTGGAGCGATTTGTCTGGTT, CGCTGAGCCAGTCACTGTAG, 200 bp.

Data mining

Our raw data are deposited in the GEO repository (GSE3632). The TIGR Multiexperiment viewer (Tmev version 2.2, <http://www.tm4.org>) was used for (1) unsupervised hierarchical clustering (UHC) using the average dot product and complete linkage options, (2) evaluation of sample re-assignment by a jackknife procedure (1000 iterations), and (3) supervised classification with the Significance Analysis of Microarrays (SAM) tool that selects discriminating transcripts^[18] (our parameters were adjusted to an FDR < 1%). Supervised classification by Support Vector Machine (SVM) was done as indicated (<http://svm.sdsc.edu/>). The Onto-Express program (<http://vortex.cs.wayne.edu>) and the FatiGO program set to level 4 or more (<http://fatigo.bioinfo.cnio.es/>) were used to categorize mRNA/protein function(s) by ontology. Detailed protein functions were retrieved with the SOURCE (<http://genome-www5.stanford.edu/cgi-bin/source/sourceSearch>) and/or OMIM (<http://www3.ncbi.nlm.nih.gov/entrez/query.fcgi?db=OMIM>) tools, and protein networks were identified with Bibliosphere (www.genomatix.de). Gene locations on chromosomes were found in Onto-Express. Statistical analysis was carried out with the GraphPad Instat software, version 3 (<http://www.graphpad.com/>). Differences in transcript levels between groups were evaluated with Mann and Whitney's non parametric test. Significant differences in the numbers of functionally-related mRNAs that were differently regulated in tumor *vs* cirrhosis as a function of etiology were evaluated from 2 × 2 tables (number of mRNAs in a given functional subset *vs* number of all other mRNAs with other functions, in HCV *vs* alcoholic patients) by Chi square test (with Yates' correction, when required). Likewise, significant differences in the chromosomal locations of dysregulated genes as a function of etiology were evaluated from a 2 × 2 table per chromosome (number of dysregulated genes on this chromosome *vs* total number of other dysregulated genes, in HCV *vs* alcoholic patients) or arm (number of dysregulated genes on this arm *vs* number of all other *Liverpool* genes on this arm, in HCV *vs* alcoholic patients).

RESULTS

Some genomic and functional features of HCC/cirrhosis are etiology-dependent

In Table 1 various features are detailed for 35 patients with HCC (18 alcoholic patients A2-A34; 17 HCV patients V1-V35; no HBV-positive patients), and 5 HCC-free controls (C1 to C5). No clinical parameter differed between our alcoholic *vs* HCV groups. These groups were randomly separated into training (V1-A22) and test set (V23-A34). The number of mRNAs detected by microarray in the training set was 7617 ± 1270 (mean ± SD in controls), 7225 ± 1586 (tumors), or 6955 ± 1644

(cirrhosis), in keeping with a trend to down-regulation in HCC^[7]. A comparison of transcript levels between tumor and surrounding cirrhosis selected a number of transcripts that could separate 3 major clusters by UHC, namely tumors, cirrhosis, and controls, as expected^[2,4-6,10] (Figure S1, available on the wjg website).

We sought for transcripts with etiology-associated differences and this was first done regardless of the source, i.e. tumor *vs* cirrhosis. Using a pair-wise ratio (transcript level in tumor/transcript level in cirrhosis) resulted in 2730 transcripts with an abnormal ratio in at least 1 patient. Dysregulated transcripts were then limited to those with an abnormal ratio in at least 3 patients from at least 1 etiology group. This empirically determined cut-off was a compromise between a lower figure that provided many non-informative transcripts of a higher figure that selected too few transcripts (not detailed), and this resulted in 475 dysregulated transcripts. Because clusters of tissue-dependent genes can be co-regulated by chromosomal co-localization^[19], we investigated whether some of these 475 genes were co-localized on given chromosome(s). Indeed, a higher number of dysregulated genes on chr.2 along with a lower number of dysregulated genes on chr.8 (supplemental Table S1 available on the wjg website) were found in HCV *vs* alcoholic patients (Figure 1). These etiology-dependent differences in gene location were still found when separately considering the p arm ($P < 0.01$) or q arm ($P < 0.01$) of chr.2 and -8. Remarkably, abnormalities on chr.2 and -8 have been previously associated with HCC (see Discussion), which indirectly supports our cut-off above and etiology-dependent findings.

Within the above 475 transcripts we next searched for prominent protein functions as identified by ontology, and 283 transcripts with such functional information could be retrieved (further details in Table 2, footnote 3). As shown in Table 2, a significantly increased frequency and expression [(tumor/cirrhosis) ratio] of dysregulated transcripts coding for cell cycle regulation or transcription factors was found in the HCV patients. Proteins of the cell cycle were mainly activators of cyclin-dependent kinase phosphorylation (CDC37, CKS2), microtubule-associated proteins (CCT4, MAPRE1), a negative regulator of the G1/S transition (CUL1), a proliferation-associated c-myc activator (NME1) and a tumor suppressor (TSC1). Several transcription factors were directly relevant to a defence of the tumoral hepatocyte following HCV infection. Although the difference between tumor and cirrhosis was not always significant, in HCV patients the tumor/cirrhosis ratios for IRF3 and SPIB, two interferon alpha and beta activators, were increased whereas the ratio for IRF6, an as yet unclear regulator of interferon production, was decreased and that of IRF2, a repressor of interferon synthesis, remained close to 1. Also, the ratio for the repressor ATF3 that targets many viral promoters was increased.

On the opposite, an increased frequency of dysregulated transcripts for plasma proteins of the acute phase response was found in the alcoholic patients. In these patients, the tumor/cirrhosis ratio of these transcripts was indicative of an inflammatory condition as it was increased (CRP, ORM1, SAAs) or decreased

Table 1 Clinical data from patients with HCC or controls

Patient ¹	Sex	Age	Pathology	Etiology ²	Tumor grade ³	Number of nodules ⁴	Nodule size (cm)	Vascular invasion	Lymphocyte infiltration ⁵
V1	F	71	HCC	HCV	3	2	4; 5	Yes	0
V3	F	67	HCC	HCV	3	1	2.5	No	+++
V4	F	72	HCC	HCV	3	1	4	No	+++
V8	M	66	HCC	HCV	3	1	4	Yes	++
V9	M	65	HCC	HCV	3	2	1.5; 3	No	+
V14	M	63	HCC	HCV	2	1	2	No	0
V15	M	70	HCC	HCV	1	1	3.5	No	+++
V17	M	69	HCC	HCV	3	1	2.5	No	+++
V20	F	73	HCC	HCV	3	1	1.5	Yes	++
V21	M	65	HCC	HCV	2	1	2	No	+
V23	F	68	HCC	HCV	2	2	1; 5.5	Yes	0
V24	M	80	HCC	HCV	1; 3	2	2.5; 3.5	No	0
V25	M	64	HCC	HCV	3	2	2; 5.5	Yes	+++
V26	M	46	HCC	HCV	3	1	2.5	Yes	+
V27	M	55	HCC	HCV	3	2	3; 3.5	Yes	0
V28	M	55	HCC	HCV	2	2	4; 4	No	++
V35	M	53	HCC	HCV	2; nd; nd	3	7; nd; nd	Yes	+
A2	M	68	HCC	ALC	2	1	11	No	0
A5	M	79	HCC	ALC	2	1	6	No	++
A6	M	63	HCC	ALC	2	1	3	No	+
A7	M	49	HCC	ALC	1	1	2	No	0
A10	M	73	HCC	ALC	2	1	5	No	+
A11	M	50	HCC	ALC	2	1	4.5	No	+
A12	M	64	HCC	ALC	1	1	2.5	No	+
A13	M	72	HCC	ALC	3	1	2.5	Yes	++
A16	M	56	HCC	ALC	3	1	8.5	Yes	++
A18	M	70	HCC	ALC	2	1	3.5	No	+
A19	M	70	HCC	ALC	3	1	2.5	No	+++
A22	M	78	HCC	ALC	1	1	1.7	No	+
A29	M	66	HCC	ALC	2	1	7	No	+
A30	M	55	HCC	ALC	2	1	4.5	No	0
A31	M	56	HCC	ALC	2	1	4	Yes	0
A32	F	66	HCC	ALC	3	1	8	No	0
A33	M	55	HCC	ALC	3	1	15	Yes	0
A34	M	69	HCC	ALC	3	1	2.2	Yes	0
C1	M	74	AD ⁶	-					
C2	F	45	AD	-					
C3	F	68	AD	-					
C4	F	43	AD+FNH ⁶	-					
C5	F	30	FNH	-					

¹V1 to A34, patients with cirrhosis and HCC, A or V refers to the alcoholic or viral etiology. C1 to -5, control patients. Patients V1 to V21 and A2 to A22 were first studied by microarray, and next used as a training set for SVM. ²HCV: Hepatitis C virus infection; ALC: Alcohol abuse; -: None. ³Differentiation grade; nd: Not determined. ⁴In multinodular HCCs, only 1 nodule was studied. ⁵Nodular infiltration; semi-quantitative appraisal done by a trained anatomopathologist (AF). ⁶Histologically normal liver taken away from a benign adenoma (AD) or a focal nodular hyperplasia (FNH).

(AHSR) in keeping with known regulation in an acute phase response^[16]. Surprisingly, the ratio for the anti-inflammatory glucocorticoid receptor NR3C1 was concomitantly increased, possibly as an attempt to limit the extent of this inflammation. Conversely, in HCV patients the ratios for APCS and CRP (acute phase plasma proteins), FOSL2 (a member of the Jun/Fos family that regulates some acute phase genes) and ETS2 (an up-regulator of inflammation) were decreased and the ratio for DSIPI (an anti-inflammatory transcription factor) was increased. Taken together, our data argued for a significant inflammatory condition in tumor as compared to adjacent cirrhosis in alcoholic but not HCV patients.

Finally, a tight SAM selection made from the 2730 transcript ratios above identified 23 non-redundant transcripts (29 probes) whose higher ratio in alcoholic *vs* HCV patients was statistically significant (red dots in Figure 2A). No transcript with a decreased ratio could be

identified, but such an imbalance in informative transcripts is not unusual with SAM^[18]. Using these 23 transcripts in UHC separated the 22 HCV and alcoholic patients into two etiology-associated groups (Figure 2B). Only 2 patients were misclassified (A18, V15) which was further evaluated by a jackknife procedure (Figure 2B legend).

The etiology-dependent transcriptomes are found in the HCC nodules

We next investigated whether the etiology-associated differences in transcript levels depended on the transcript source, i.e. tumor *vs* cirrhosis. When using the (transcript level in tumor/mean transcript level in controls) ratio in nodules, a total of 2641 transcripts had a significantly abnormal ratio in at least 1 of our patients 1-22 (black and red dots in Figure 3A). SAM identified 18 non-redundant transcripts whose (tumor/controls) ratio was significantly higher in alcoholic *vs* HCV patients (red dots in Figure 3A).

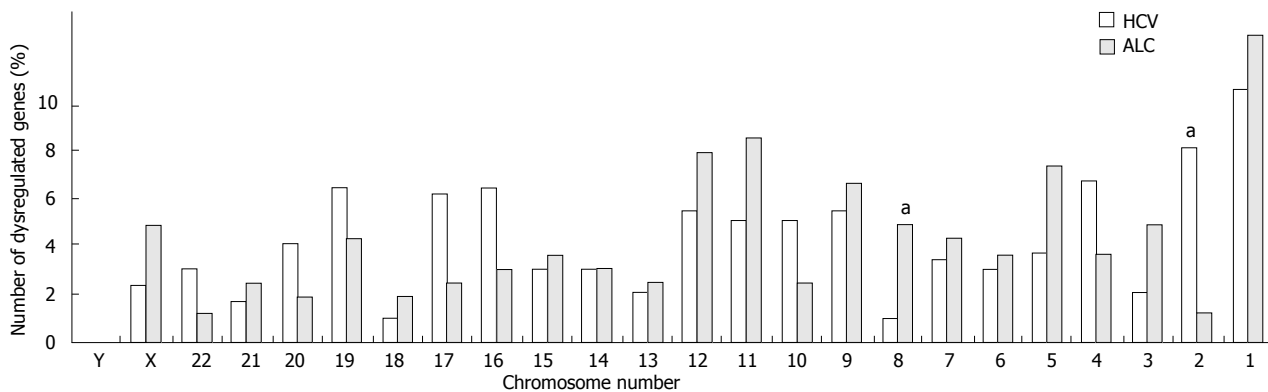


Figure 1 Etiology-dependent location of dysregulated genes. A total of 475 genes (HCV, 301 genes; alcoholism, 174 genes) with a dysregulated transcript were studied. Dysregulated transcripts were defined by an abnormal (tumor/cirrhosis) ratio in at least 3 patients of at least one etiology group (see details in Table 2, footnote 3). The number of dysregulated genes per chromosome is expressed as a percentage of the total number of dysregulated genes per etiology. Significant differences of gene frequency on a given chromosome in HCV vs alcoholic patients are: chr 2, $P = 0.004$; chr 8, $P = 0.02$ (Chi square test with Yates' correction), $^aP \leq 0.02$.

Table 2 Etiology-dependent frequency of dysregulated transcripts within functionally defined subsets

HCV patients ($n = 10$)			Alcoholic patients ($n = 12$)		
		Tumor/cirrhosis ¹			Tumor/cirrhosis
Subset: Regulation of cell cycle (GO 0000074) ²					
CCT4 ³	Hs.421509 ⁴	1.38 (7) ^a			
ZAK	Hs.444451	1.44 (8)			
CUL1	Hs.146806	1.69 (7)			
MAPRE1	Hs.472437	1.78 (10)			
CLK1	Hs.433732	1.80 (7)			
CKS2	Hs.83758	1.92 (8)			
NME1	Hs.463456	1.97 (9)			
CDC37	Hs.160958	2.05 (6)			-
TSC1	Hs.370854	2.60 (6) ^a			
Subset: Transcription factor (GO 0003700)					
IRF6	Hs.355827	0.55 (10) ^b	RUNX1	Hs.149261	0.67 (12) ^b
FOSL2	Hs.220971	0.58 (10) ^a	NR3C1	Hs.122926	1.68 (12) ^b
ETS2	Hs.592158	0.79 (10) ^b			
IRF2	Hs.374097	0.83 (9)			
DSCR1	Hs.282326	1.53 (10)			
NR4A3	Hs.279522	1.63 (7) ^a			
ZNF397	Hs.84307	1.72 (10)			
HMGA1	Hs.518805	1.75 (9)			
SPIB	Hs.437905	1.77 (10)			
NME1	Hs.463456	1.97 (6) ^a			
ATF3	Hs.460	2.22 (6)			
MSRB2	Hs.461420	2.32 (6)			
DSIP1	Hs.420569	2.51 (7)			
IRF3	Hs.75254	2.70 (7)			
Subset: Acute phase response (GO 0006953)					
CRP ⁵	Hs.76452	0.46 (10) ^a	AHSG	Hs.324746	0.84 (12)
APCS ⁵	Hs.507080	0.84 (10) ^a	ORM1	Hs.567311	1.24 (12)
			SAA2 ⁵	Hs.1955	1.84 (11) ^a
			SAA1 ⁵	Hs.632144	1.90 (11) ^a
			CRP ⁵	Hs.76452	2.17 (12)

¹Average ratio of mRNA levels in tumor and cirrhotic tissue in patients with a detectable expression of this mRNA. The number of such patients is indicated in brackets and a significant difference between tumors and paired cirrhotic tissues per etiology is indicated (^a $P < 0.05$; ^b $P < 0.01$; Wilcoxon's non parametric, paired test). ²Subset of mRNAs coding for proteins with a shared function as identified by Gene Ontology (GO) number. ³Dysregulated mRNAs, as defined by an abnormal tumor/cirrhosis ratio found in at least 3 patients of at least one etiology group (total 475 mRNAs; HCV, 301; alcoholism, 174). The dysregulated mRNAs with an ontology-defined function (HCV, 183; alcoholism, 100) were a subset of the 475 mRNAs. ⁴Hs. number as a unique mRNA identifier. ⁵Only 5 of the 23 mRNAs listed in Figure 2B appear herein because the other mRNAs in Figure 2B belonged to functional classes in which the number of mRNAs was not different in alcoholic vs HCV patients.

As shown in Figure 3B, UHC of our 22 patients as based upon these 18 ratios provided two major clusters of HCV- or alcohol-associated HCCs. Only 2 samples were

misclassified (V-TU14, V-TU15), which was confirmed by a jackknife procedure (Figure 3B legend). Interestingly, all controls were clustered with the alcoholic tumors (right

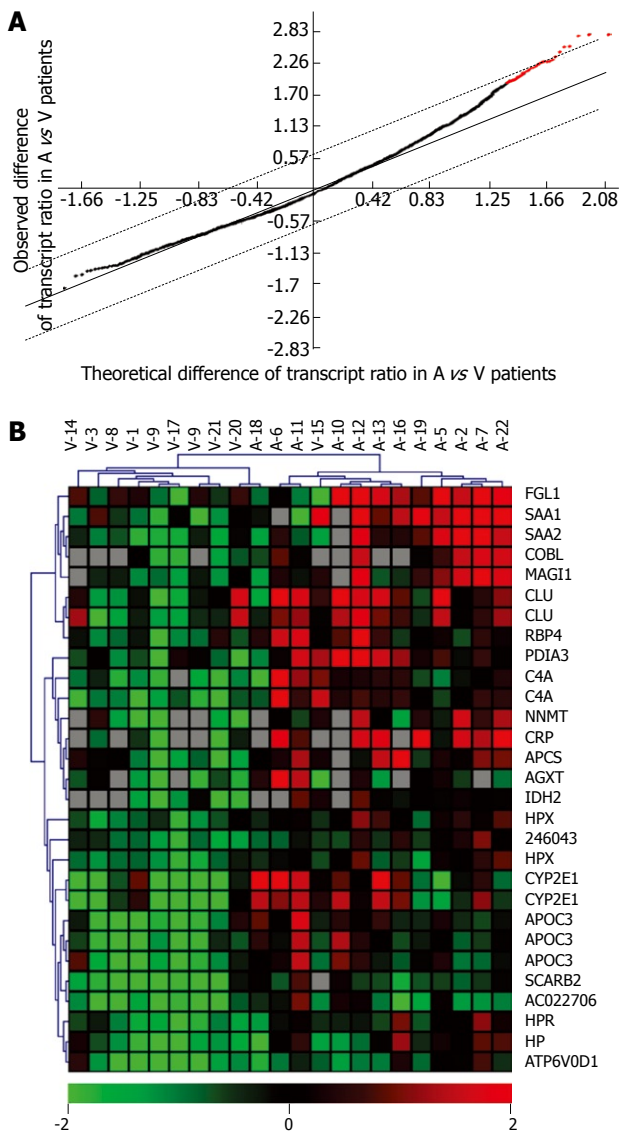


Figure 2 Etiology-dependent clustering of patients with (tumor/cirrhosis) ratios. **A:** Selection of transcripts with a significantly abnormal ratio of abundance in (tumor/paired cirrhosis). Black + red dots: 2730 transcripts with an abnormal ratio in at least 1/22 patients. Red dots: 23 informative transcripts (29 probes) with a significantly increased ratio in alcoholic vs HCV patients as selected by SAM with an FDR < 1%. **B:** UHC of 22 HCC patients with the above 23 transcripts. Note that the misclassification of two samples (A18, V15) was further evaluated by a jackknife procedure (1000 iterations) which supported cluster assignment to a variable extent (A18, < 50%; V15, 100%). Note that several transcripts were each detected with 2 or more different cDNA probes. Bottom scale bar: decreased (green), increased (red) or identical ratio (black). Gray squares are missing values. All data given on a log₂ scale.

side of Figure 3B), thus suggesting that alcoholism may alter expression of these 18 transcripts to a lesser extent than HCV infection.

Because the etiology-dependent transcripts identified from (nodule/cirrhosis) ratios (Figure 2) or from (nodule/controls) ratios (Figure 3B) largely overlapped (15/18 transcripts; 83%; stated in Figure 3B), this suggested that the nodules could be responsible for transcript abnormalities. Indeed, SAM made with the ratios from cirrhotic samples, i.e. (transcript level in cirrhosis/mean transcript level in controls), failed to identify a series of etiology-discriminant transcripts (no red dots in Figure 3C). Moreover, and as shown in Figure 3D, no etiology-

dependent clustering of patients V1-A22 was obtained when using the (cirrhosis/controls) ratios for these 18 stated transcripts. We concluded that such transcript levels now appear to be an etiology-dependent variable in nodules, but not in cirrhosis.

Taken together, our data indicated that a subset differently dysregulated following HCV infection *vs* alcoholism can be identified in cancerous nodules whereas it cannot be detected at an earlier stage of hepatic dysplasia, namely the surrounding cirrhosis.

Data validation

Our data above were validated with a conceptually different tool (qRTPCR). After excluding 3 transcripts whose gene structure was unknown (AC022706, FLJ33814, 246043) all transcripts previously found to vary significantly with etiology (i.e. cumulated lists from Figure 2B and 3B, total 23 transcripts) were quantified in every HCC patient in this study. Given our previous observation that cirrhotic samples were not informative, these transcripts were measured in tumors only. First, the informativeness of each of the above 23 transcripts was tested in our entire population of 35 HCC samples. As shown in Figure 4A, most transcripts (18/23, 78%) were significantly overexpressed in alcoholic tumors, as expected from Figure 3A. Next, UHC reproducibly resulted in a perfect and etiology-dependent separation of our test set (V23-V35 and A29-A34) (Figure 4B). Finally, a classification algorithm generated with qRTPCR data from the training set (V1-A22) by SVM separated our test set into etiology-dependent groups with 2 misclassifications (V23, V24).

As for protein functions the increased level of CYP2E1 transcript in alcoholic patients fits its known up-regulation by ethanol^[20]. Most other proteins in alcoholic patients were associated with the inflammatory response, as inferred from a comparison with our earlier data^[16] as well as putative protein relationships retrieved with Biblosphere (data not shown). They included acute phase proteins (APCS, APOC3, C4A, CRP, HP, HPX, NNMT, RBP4, SAAs) whose directions of variations indicated a stronger inflammatory condition of the tumor in alcoholic *vs* HCV patients. Remarkably, most of these acute phase proteins are scavengers of endogenous toxicants or protect against membrane peroxidation (CRP, HP, HPX, RBP4, SAAs). Among them, heme detoxicants include two hemoglobin transporters (HP, HPR) and one heme scavenger (HPX), whose variations suggested a higher iron metabolism in alcoholic *vs* HCV tumors. In alcoholism-associated tumors, only two proteins were associated with cell proliferation (FGL1) and apoptosis limitation (CLU).

DISCUSSION

Transcriptome-wide analysis in alcoholism-induced HCC has seldom been studied, which has prevented its detailed comparison with other etiologies. Indeed, transcript alterations in alcoholism-associated HCC have been reported but they focused on a limited number of transcripts or they did not differentiate between cirrhotic and cancerous tissues^[21,22]. In contrast, we have now compared transcripts in tumor *vs* paired cirrhosis

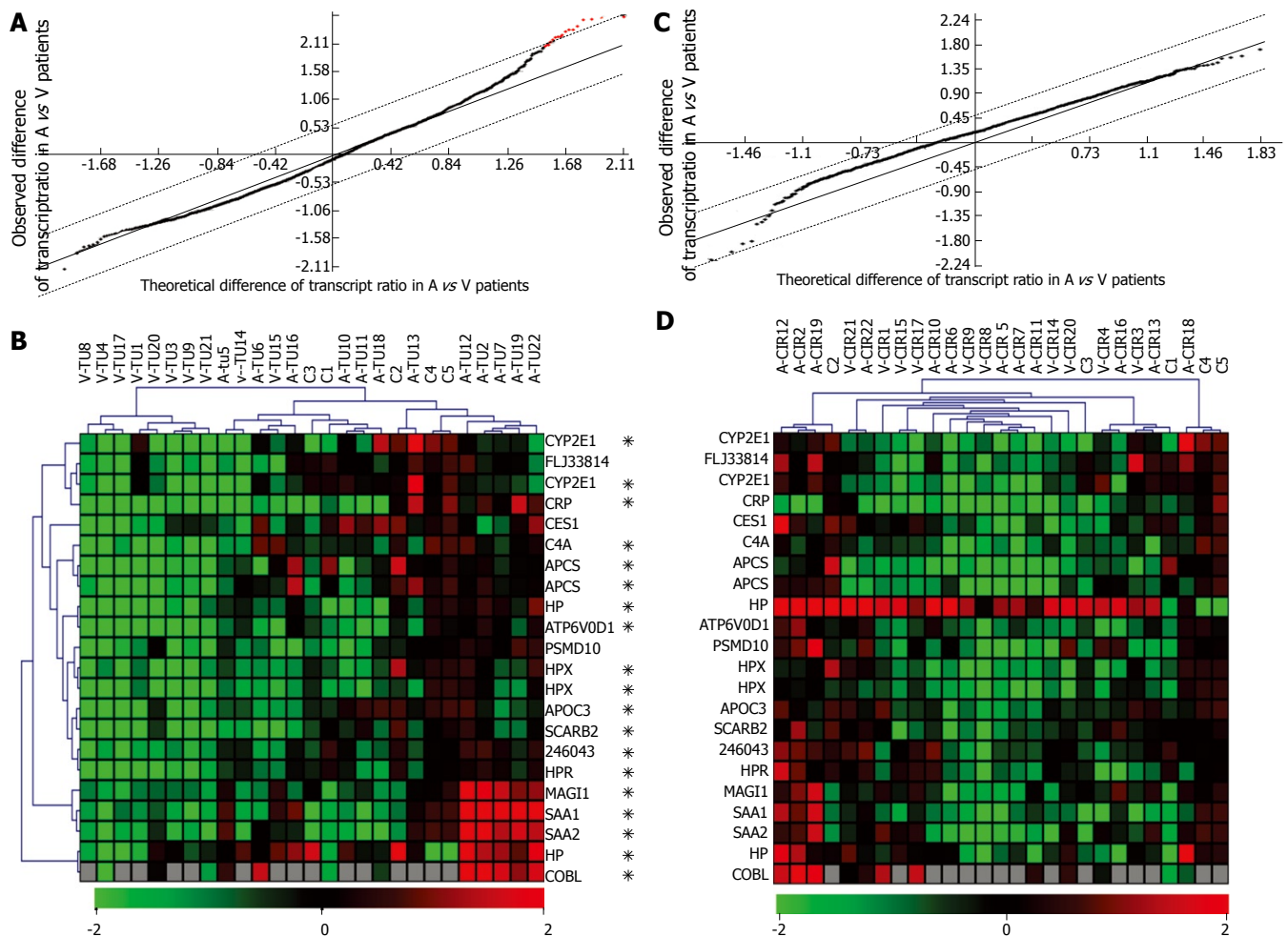


Figure 3 Etiology-dependent clustering of tumors only, with (tumor/controls) ratios. **A:** Selection of transcripts with a significantly abnormal (level in tumor/mean level in controls) ratio. Black + red dots: 2641 transcripts with an abnormal ratio in at least 1/22 patients. Red dots: SAM selection of 18 informative transcripts (22 probes) with a significantly increased (tumor/controls) ratio in alcoholic vs HCV patients. SAM parameters as in Figure 2; **B:** UHC of 5 control livers and 22 tumors from 22 HCC patients, as done with the data of the 18 informative transcripts selected in **A**; The stars point to 15 transcripts (19 probes) that also belonged to the set of 23 transcripts previously selected in Figure 2; **C:** Selection of transcripts with a significantly abnormal (level in cirrhosis/mean level in controls) ratio. Black dots: 2037 transcripts with an abnormal ratio in at least 1/22 patients. No informative transcript was selected by SAM (no red dot); **D:** UHC of 5 control patients and 22 HCC-associated cirrhotic samples from 22 HCC patients, as done with the list of 18 transcripts used in **B**. Other details as in Figure 2.

by searching for abnormal (tumor/cirrhosis) ratios at a genome-wide level. We used a non-stringent selection of 475 transcripts whenever wide groups of transcripts were required (chromosomes; ontology) but we used SAM with a tight FDR to identify a total of 23 transcripts with strong etiology-associated variations. Second, we compared tumors (or cirrhosis) *vs* controls, which identified 18 transcript alterations in tumors only. The combined levels of these 18 transcripts in tumors but not in paired cirrhosis could classify the patients by etiology. Interestingly, most of these transcripts were previously associated with cirrhosis and HCC, this including, for instance, APCS, APOC3, CLU, CRP, CYP2E1, FGL1, HP, HPX, NNMT, RBP4, SAAs, and their down-regulations, mainly seen in an HCV context, were consistent with our present data^[14,23-25]. The present lack of an etiology-dependent transcriptome in the cirrhosis surrounding HCC indicates that some etiology-dependent mechanisms take place at a relatively late stage of tumoral transformation. Further analysis of HCC-free cirrhosis will clarify this stepwise process.

As inferred from ontology, cell cycle regulation and a response to interferons appear to predominate in HCV-associated tumors. Our observation that over-expression of interferon-responsive genes in tumor *vs* cirrhosis is restricted to HCV patients also fits this viral etiology, as well as the ethanol-induced down-regulation of interferon gamma signalling in hepatoma cells^[26]. HCCs with an alcoholic or viral origin have been subgrouped by others as a function of activation or repression of interferon-regulated genes but etiology influence was not documented^[6]. The potent response of the normal hepatocyte to interferons is repressed by an HCV infection^[27]. Therefore, the over-expression of interferon-responsive genes in nodule *vs* cirrhosis, as seen herein, suggests that a repression of these genes occurs in cirrhosis but escapes, at least partly, this viral mechanism in nodules.

The iron overload/HCC association is well established. Non transferrin-bound, free iron is carcinogenic and facilitates tumor growth *via* the production of ROS and free radicals, and subsequent lipid peroxidation^[28]. Free iron and ROS in hepatocytes are a side effect of chronic

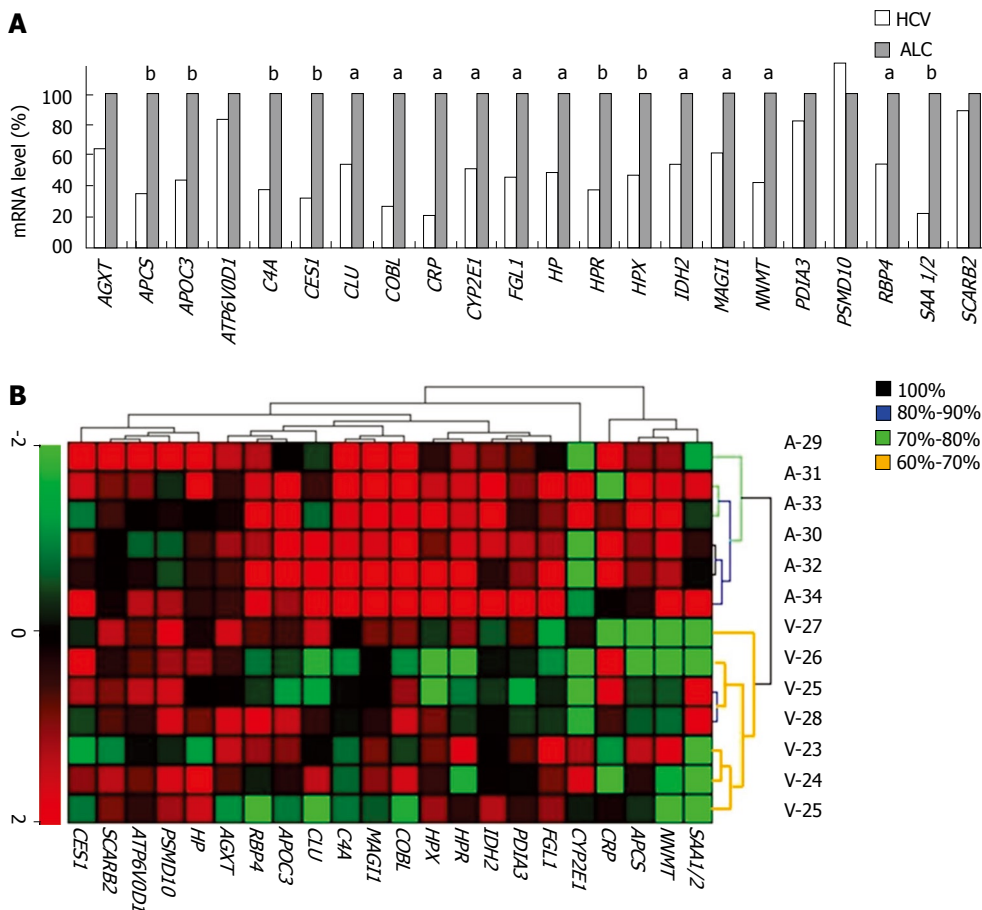


Figure 4 Data validation by qRT-PCR of tumor mRNAs. The levels of 23 transcripts (listed from Figures 2A and 3A; SAA1 and -2 are counted as 2 transcripts) were determined in tumors and normalized with the 18S RNA level. **A:** Every histogram depicts the mean transcript level in all patients from Table 1 ($n = 35$) and is expressed as a percentage of the mean level in alcoholic patients (100%). Mann and Whitney's test ($^aP < 0.05$; $^bP < 0.01$). **B:** UHC made with qRT-PCR data from our test set ($n = 13$). The colors in dendrogram indicate the percentage of iterations reproducibly providing the same separation. Note the significant separation of 2 major, etiology-related branches (black branches found in 100% of 10^3 iterations).

alcoholism^[29]. Our ontology-based data, up-regulations and functions of SAM-selected transcripts indicated that in alcoholic nodules an acute phase response is a prominent event. Therefore, a high extent of inflammation could participate in an etiology-dependent antitumoral response of the hepatocyte. However, this view is now challenged when considering (1) the induction of an inflammatory response in liver following both alcoholism and HCV infection^[6,30,31], (2) the similar extent of lymphocyte infiltration in both etiologies in our patients, (3) the limited apoptosis in alcoholic tumors and, most importantly, (4) the restricted functions of the afore mentioned set of acute phase transcripts. Indeed, in alcoholic patients the up-regulated levels of acute phase transcripts point to acceleration of iron metabolism (HP, HPX), a detoxication mediated by the haemoglobin degradation pathways (HP, HPR, HPX), and a protection against membrane peroxidation (CLU, CRP, RBP4, SAAs), and hence they strongly suggest accelerated exchange of free- *vs* bound iron in nodules. If so, the increase in proteins that prevent membrane peroxidation (CLU, CRP, RBP4, SAAs) represents a concomitant against free iron. Overall, we propose a set of transcripts as indicators of a detrimental iron metabolism whose extent and control are etiology-dependent. Alteration of this metabolism in alcoholism-induced tumors, as now suggested by the high levels of relevant transcripts, could participate in the free iron limitation noticed in HCC nodules^[32].

An increasing number of chromosome amplifications, mutations, deletions and transpositions develop during the transition from preneoplasia to HCC^[1]. Such events on

chr.8 have allowed discrimination of patients with beta-catenin mutations and an allelic loss of chr.8p only from patients with a heterogeneous series of gains/losses of various other chromosome segments^[9]. In contrast, chr.2 abnormalities are infrequent in HCCs^[9]. Our present work now establishes a link between etiology and an abnormal expression of various genes on chr.2 (HCV) or -8 (alcoholism). This conclusion based upon transcript levels will require further investigations of etiology-dependent structural or epigenomic alterations on these chromosomes. As our data indicate that abnormal gene expressions are spread on both arms of chr.2 and -8, aberrant methylation of a series of promoters along a chromosome segment, long range epigenetic silencing and/or abnormal copy numbers of a chromosome^[33-35] could explain our observations.

Overall, our data point to major etiology-associated differences in HCC. Given that HCC therapies have not yet considered any etiology-dependent mechanisms of carcinogenesis, our observations open new avenues for therapies that should take into account HCC etiology.

COMMENTS

Background

Chronic hepatitis C virus (HCV) infection and alcoholism are two important causes for hepatocellular carcinoma (HCC). Liver transcriptome analysis has resulted in the identification of genes with an aberrant expression according to different physiopathological states. In the present work, we performed a comparison of liver transcriptomes in HCV virus- vs alcoholism-associated HCC.

Research frontiers

Table S1 Etiology-dependent location of dysregulated genes on chromosomes

HCV			Alcohol		
gene	Hs. Number	chr.	gene	Hs. Number	chr.
Trans. Locus	Hs.597833	2	MAP3K2	Hs.145605	2q14.3
VAMP5	Hs.172684	2p11.2	WDFY1	Hs.642721	2q36.1
KCMF1	Hs.345694	2p11.2			
LOC56902	Hs.262858	2p13.3			
UGP2	Hs.516217	2p14-p13			
CCT4	Hs.421509	2p15			
KIAA1387	Hs.516182	2p16.1			
MRPL33	Hs.515879	2p21			
FNDC4	Hs.27836	2p23.3			
FOSL2	Hs.220971	2p23.3			
CKAP2L	Hs.434250	2q13			
CXCR4	Hs.421986	2q21			
ZAK	Hs.444451	2q24.2			
LEREPO4	Hs.368598	2q32.2			
CLK1	Hs.433732	2q33			
MAP2	Hs.368281	2q34-q35			
CPS1	Hs.149252	2q35			
NHEJ1	Hs.225988	2q35			
WDFY1	Hs.642721	2q36.1			
AGXT	Hs.144567	2q36-q37			
TNRC15	Hs.565319	2q37.1			
DGKD	Hs.471675	2q37.1			
STK25	Hs.516807	2q37.3			
LONRF1	Hs.180178	8p23	PLAT	Hs.491582	8p12
EXOSC4	Hs.632041	8q24.3	CLU	Hs.436657	8p21-p12
TIGD5	Hs.71574	8q24.3	FGL1	Hs.491143	8p22-p21.3
			MCM4	Hs.460184	8q11.2
			FAM92A1	Hs.125038	8q22.1
			EXOSC4	Hs.632041	8q24.3
			TIGD5	Hs.71574	8q24.3
			RPL8	Hs.178551	8q24.3

The genes shown here are those located on the significant chrs in Figure 1. They are referred to by an Hs. number as a unique identifier. Every such gene had an mRNA level that was significantly altered in tumor vs cirrhosis in at least 25% of the patients within at least one etiology.

The hepatitis B virus (HBV)- or HCV-induced genetic alterations are known to be different and the associated transcriptomes have proven to vary significantly. Therefore, deciphering the transcriptome patterns as a function of HCC etiology is of critical importance. However, the gene dysregulations in a context of alcohol abuse are poorly understood and the associated transcriptome has seldom been studied. *A fortiori*, a comparison of liver transcriptomes in HCV virus- vs alcoholism-associated HCC has never been done.

Applications

Given that HCC therapies have not yet considered any etiology-dependent mechanisms of carcinogenesis, our observations open new avenues for therapies that should take into account HCC etiology.

Peer review

The manuscript by Derambure *et al.* describes a study that compared microarray data from hepatocellular carcinoma as a result from alcohol or hepatitis C. Interestingly, the authors found etiology-specific alterations in gene expression between the two HCC. These data would lead to a better understanding of the molecular basis of these disease states.

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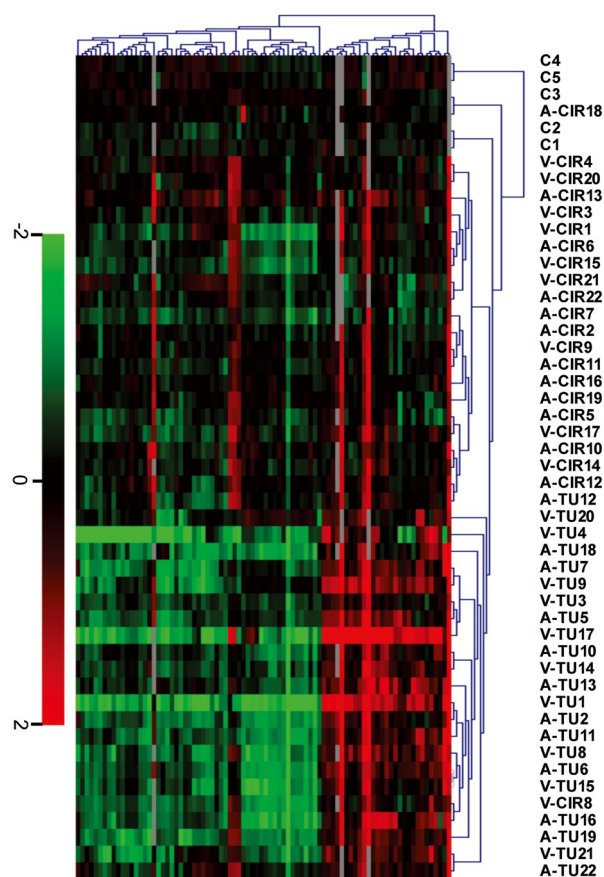


Figure S1 Sample clustering: Tumor vs cirrhosis. Unsupervised hierarchical clustering of 5 control livers (C) and paired HCC nodule (TU) and surrounding cirrhosis (CIR) (44 samples from patients 1 to 22, see clinical data in Table 1) shown from left to right was based upon 81 transcripts (84 probes) shown from top to bottom. Transcript levels were expressed as a ratio [level in sample/mean level in controls] and 81 transcripts were next selected as informative transcripts by SAM. The patients are listed on top (V, HCV; A, alcoholism). Scale bar (log2 ratio): decreased (green), increased (red) or identical mRNA level (black) in any sample vs controls. Gray squares are missing values.

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Aberrant activation of nuclear factor of activated T cell 2 in lamina propria mononuclear cells in ulcerative colitis

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is implicated in an auto-regulatory positive feedback loop of sustained T-cell activation and NFAT proteins play key roles in the calcium/calcieneurin signaling pathways, our results not only provide new insights into the mechanism for sustained intractable inflammation, but also suggest the calcium-calcieneurin/NFAT pathway as a new therapeutic target for ulcerative colitis.

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Key words: Nuclear factor of activated T cells; Ulcerative colitis; Inflammatory bowel disease; Nuclear factor of activated T cells c1; Nuclear factor of activated T cells 2

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Abstract

AIM: To investigate the role of nuclear factor of activated T cell 2 (NFAT2), the major NFAT protein in peripheral T cells, in sustained T cell activation and intractable inflammation in human ulcerative colitis (UC).

METHODS: We used two-dimensional gel-electrophoresis, immunohistochemistry, double immunohistochemical staining, and confocal microscopy to inspect the expression of NFAT2 in 107, 15, 48 and 5 cases of UC, Crohn's disease (CD), non-specific colitis, and 5 healthy individuals, respectively.

RESULTS: Up-regulation with profound nucleo-translocation/activation of NFAT2 of lamina propria mononuclear cells (LPMC) of colonic mucosa was found specifically in the affected colonic mucosa from patients with UC, as compared to CD or NC ($P < 0.001$, Kruskal-Wallis test). Nucleo-translocation/activation of NFAT2 primarily occurred in CD8+T, but was less prominent in CD4+ T cells or CD20+B cells. It was strongly associated with the disease activity, including endoscopic stage ($\tau = 0.2145$, $P = 0.0281$) and histologic grade ($\tau = 0.4167$, $P < 0.001$).

CONCLUSION: We disclose for the first time the nucleo-translocation/activation of NFAT2 in lamina propria mononuclear cells in ulcerative colitis. Activation of NFAT2 was specific for ulcerative colitis and highly associated with disease activity. Since activation of NFAT2

Shih TC, Hsieh SY, Hsieh YY, Chen TC, Yeh CY, Lin CJ, Lin DY, Chiu CT. Aberrant activation of nuclear factor of activated T cell 2 in lamina propria mononuclear cells in ulcerative colitis. *World J Gastroenterol* 2008; 14(11): 1759-1767 Available from: URL: <http://www.wjgnet.com/1007-9327/14/1759.asp> DOI: <http://dx.doi.org/10.3748/wjg.14.1759>

INTRODUCTION

Inflammatory bowel disease (IBD), which includes the two components of Crohn's disease (CD) and ulcerative colitis (UC), is a chronic, relapsing and debilitating idiopathic inflammation of the gastrointestinal tract. UC is characterized by crypt abscesses and ulceration of the colon, which rarely extend beyond the muscularis layer. In comparison, CD is characterized by granulomatous inflammation involving the whole layer of bowel wall and, by its extension, any portion of the gastrointestinal tract. Although the exact etiologies remain uncertain, there is compelling evidence suggesting the implication of environmental risk factors^[1], including commensal bacteria^[2,3], genetic predisposition^[4-9], and disturbance of the immune reaction^[10-12].

In both UC and CD, the inflamed tissue is heavily infiltrated with inflammatory cells, mainly T lymphocytes^[11,13,14]. These cells are thought to be activated and secrete large amount of cytokines, which in turn play a primary role in the pathogenesis of the diseases^[15]. In addition, based on

studies of human tissue and animal models, it has been shown that UC and CD have distinct profiles of cytokine production. UC predominantly presents with type 2 helper T cell (TH2) cytokine profiles, such as IL-4, IL-5, and IL-13, while in DC, there is primarily a secretion of type 1 helper T cell (TH1) cytokines, including IL-12, IL-23, IFN- γ and TNF- α ^[12,15-20]. Recently, de-regulation of innate immunity, including defects in the mucosal barrier and in innate effector cells such as neutrophils, monocytes, and dendritic cells, has been proposed to initiate early events and perpetuate the inflammatory state in CD^[21]. However, the underlying mechanisms that initiate and promote the diverse immune perturbation in IBD remain to be determined. Moreover, elucidating the mechanisms that lead to aberrant immune activation of the colonic mucosa will contribute not only to understanding disease pathogenesis but also to the development of new therapies^[22-29].

Recently, we have used proteomic approaches to identify 19 differentially expressed proteins in the colonic mucosa of UC patients^[30]. Of these, up-expression of the nuclear factor of activated T cells c1 (NFATc1 or NFAT2) is of particular interest, since NFAT proteins play pivotal roles in the development of the cardiovascular system and the regulation of immune function. Herein, we report results demonstrating nuclear translocation and activation of NFAT2 in infiltrating lymphocytes of UC diseased colonic mucosa.

MATERIALS AND METHODS

Patients and tissue processing

Colonic tissue sections were obtained from 107 cases of UC, 15 cases of CD, 48 cases of non-specific colitis, and 5 cases of normal controls. All UC patients, CD patients and non-specific colitis patients who underwent colonic endoscopic biopsy or surgical resection from April 1984 to September 2004 at our hospital were retrospectively included in the study in accordance with the clinical and pathologic diagnosis of UC, CD, or excluding the diagnosis of UC or CD, respectively. Those who had a tentative diagnosis of UC or CD, with a course of clinical symptoms less than three months, were excluded in this study. Seventy out of the 107 UC patients who had been regularly followed-up or were deceased by the end of the follow-up period (October, 2006) were included in the association studies between NFAT2 activation and clinical presentations. Disease activity was assayed on the basis of endoscopic observation of the mucosal pattern and histological grading^[31,32].

For proteomic assays using two-dimensional gel-electrophoresis, colonic biopsy samples were taken from the inflamed mucosa in four UC patients (all males; age range 25-42 years, average age 33 years), 3 cases of non-specific, infectious colitis (males, aged 21, 28, and 43 years), and 5 individuals without obvious colonic disease (all males; age range: 24-44 years, average age 33 years). The duration of the disease for the four UC patients was 6, 4, 6, and 10 years, respectively. No patient received immunosuppressive or steroid treatment for at least three weeks before the beginning of the study. All biopsy

samples were collected from the distal portion of the sigmoid colon (15-25 cm from the anal verge) and were then cut into two parts. One was immediately frozen at -80°C for subsequent proteomic assays, while the other was fixed in fresh 4% paraformaldehyde and embedded in paraffin for histologic and immunologic staining. Diagnosis of UC was made in accordance with standard criteria. The institutional ethics committee approved all of the protocols and all enrolled patients gave their informed consent, except those whose tissue samples had been collected and stored in the tissue bank of Chang Gung Memorial Hospital before 2000.

Two-dimensional gel electrophoresis and analysis

The methods used for two-dimensional gel electrophoresis have been described previously^[1]. In brief, frozen tissue samples (200 mg) were homogenized in 2 mL homogenization buffer (50 mmol/L Tris-HCl, pH 7.2) containing a protease inhibitor cocktail (1 mmol/L 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride, 0.8 mmol/L aprotinin, 21 mmol/L leupeptin, 36 mmol/L bestatin, 15 mmol/L pepstatin A, 14 mmol/L E-64) using a homogenizer (IKA Labortechnik, Staufen, Germany) at 25000 r/min. Samples containing 120 μ g protein were subjected in isoelectric focusing using the IPG strips in Protean IEF Cell (BioRad) in accordance with the manufacturer's instructions. Separation in the second dimension of 12.5% polyacrylamide gel slabs was carried out using Protean II electrophoresis equipment (BioRad).

The gels were initially fixed in 10% methanol and 7% acetic acid, and then stained for 3 h in a commercially available SYPRO Ruby buffer (Molecular Probes, Eugene, OR). Protein patterns in the gels were recorded as digitalized images using a high-resolution scanner (GS-710 Calibrated Imaging Densitometer, Bio-Rad). Gel image matching was done with Progenesis software (Progenesis Discovery, Nonlinear Dynamics, Durham, NC).

Protein identification by mass spectrometry

Protein spots of interest were manually excised from the sypro Ruby stained 2D gels, de-stained using 50 mmol/L NH₄HCO₃ in 50% acetonitrile and dried. The protein was digested by overnight incubation at 37°C with trypsin at 5 ng/mL in 50 mmol/L NH₄HCO₃, pH 7.8, followed by extraction in 1 volume 0.1% TFA and being eluted in 1.5 μ L matrix (5 mg α -cyano 4-hydroxycinnamic acid/mL in 50% acetonitrile/0.1% TFA) for MALDI-TOF MS and MS/MS analysis. Both MS and MS/MS spectra were searched against the NCBI database, using the mascot software from matrix science (www.matrixscience.com) to identify the proteins.

Immuno-histochemical, double immunofluorescence and confocal microscopic studies

For immuno-histochemical studies, after being de-waxed and rehydrated, the tissue sections were incubated with mouse anti-human NFAT2 as primary antibodies (diluted 1:500 in PBS; Abcam, Cambridgeshire, UK) for 2 h at room temperature, followed by rinsing with PBS and incubation by an HRP-conjugated rabbit anti-mouse IgG, or Rhodamin-conjugated anti-mouse IgG

as the second antibody. HRP activity was detected using diaminobenzidine tetrahydrochloride (DAB) as substrate for 3 min in accordance with the manufacturer's instructions (BioGenex, San Ramon, CA 94583 USA).

To determine the relationship between the expression of NFAT2 and the cell types of LPMCs, we performed double immunofluorescence staining for NFAT2 and CD3 (BD, Franklin Lakes, NJ USA), CD4 (DAKO, Glostrup, Denmark), CD8 (Hytest, Turku Finland), and CD20 (DAKO, Glostrup, Denmark) on the same sections using Envision Doublestain System (DAKO, Glostrup, Denmark). Deparaffinization and microwave antigen retrieval were performed as described above. After quenching the endogenous peroxidase activity with peroxidase blocking reagent (DAKO, Glostrup, Denmark), tissue sections were incubated with mouse monoclonal anti-CD antibodies for one hour at room temperature, followed by rinsing with washing buffer and were incubated with HRP-conjugated anti-mouse antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA) for 30 min at room temperature with detection of peroxidase activity. Then the tissue sections were subjected to the second staining for NFAT2 with the sequential steps of quenching the endogenous phosphatase, incubation with anti-NFAT2 antibodies, incubation with anti-goat IgG antibodies and detection of the phosphatase activity in accordance with the manufacturer's instructions (Envision Doublestain System, DAKO, Glostrup, Denmark).

For confocal microscopy, tissue sections were de-waxed using xylene twice and rehydrated with PBS followed by blocking with goat serum (BioGenex, San Ramon, CA 94583 USA) for 10 min at room temperature. The tissue sections were then incubated for 2 h with the specific antibody against NFAT2 (1:50, ab25916, Abcam, plc), rinsed extensively three times with PBS, incubated with secondary antibody (1:200 Jackson ImmunoResearch) for 30 min at room temperature in the dark, and then counterstained with 2-(4-Amidinophenyl)-6-indolecarbamidine dihydrochloride (DAPI, Sigma-Aldrich). The sections were rinsed successively with PBS, distilled water, and ethanol, and mounted with a drop of Mowiol on a micro slide. Confocal microscopy was performed using a laser scanning spectral confocal microscope (Leica PCS ST2, Leica Microsystems, Wetzlar, Germany).

Statistical analysis

The degree of nucleotranslocation of NFAT2 was classified into five grades (I: 0%; II: < 10%; III: 10%-50%; IV: 50%-90%; V: > 90% of LPMC with nucleotranslocation of NFAT2) or low (< 50%) or high (> 50%). Comparing the degree of nucleo-translocation of NFAT2 among UC, CD and non-specific colitis patients, we used Kruskal-Wallis test (for UC, CD and NC three groups) or Mann-Whitney test (for any two unpaired groups). Associating the onset age with the degree of NFAT2 nucleo-translocation (< 50% *vs* > 50%) in UC patients, we used an unpaired two-sample *t* test. Examining the association of gender, sex, and disease duration before sampling with the degree of NFAT2 nucleo-translocation (< 50% *vs* > 50%), we used a corrected χ^2 test. To test association of the degree of nucleo-translocation of NFAT2 (< 50% *vs* > 50%) to the clinical outcome of

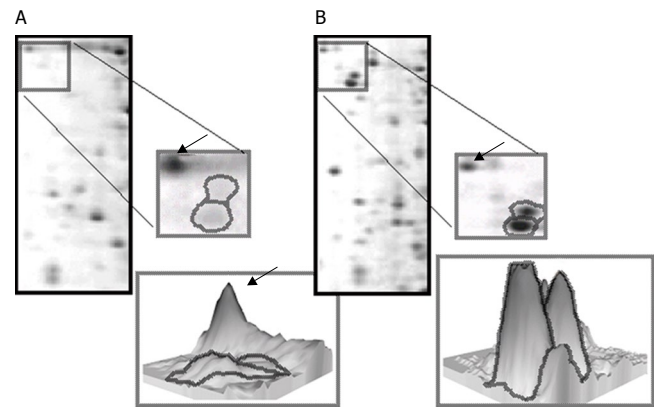


Figure 1 Two-dimensional gel electrophoresis identifying increased amounts of NFAT2 in UC colonic mucosa tissue. Tissue protein lysate was prepared from normal (A) and UC affected colon tissues (B) and separated using two-dimensional gel electrophoresis (2-DE). Spot identification and matching across the gels and determination of the relative amount for each corresponding protein spot were conducted using the software, Progenesis workstation (non-linear). Proteins were identified using mass spectrometry as previously described^[30]. The representative results of 2-DE are shown. The inserts are the close views along with the 3-dimensional pictures of NFAT2 and the reference protein spot on the 2-DE. Arrows indicate the reference protein spots across gels. The grey lines outline the NFAT2 spots.

UC patients, we used a two-side tailed Fisher's exact test. Correlating the degree of NFAT2 nucleo-translocation (grade I-V) to the disease activity (endoscopic grade 1-4 and histological grade 1-4) of UC patients, we used Kendall's τ -rank correlation coefficient.

RESULTS

Up-expression of NFAT2 in the proteomes of UC colonic mucosa

To identify the de-regulated proteins in colonic mucosa of UC, two-dimensional gel electrophoresis was used to compare the colon-mucosa proteomes between four UC patients and three healthy controls. Of interest is the up-expression of NFAT2 in UC colonic mucosa (Figure 1), because of its potential role in regulating immune function.

Nucleo-translocation/activation of NFAT2 in the mucosa infiltrating lymphocytes in UC colonic tissues

To validate the up-expression of NFAT2 in UC diseased colonic mucosa, immuno-histochemistry was used to examine the relative number of cells specifically expressing NFAT2 in colonic mucosal tissue sections obtained from healthy controls and UC and CD patients. As shown in Figure 2, NFAT2 was detected in LPMCs obtained from the UC (Figure 2 D-F) and CD patients (Figure 2 G-I), as well as from the healthy controls (Figure 2 A-C), indicating that the up-expression of NFAT2 in UC was primarily due to the increase in the number of mucosa infiltrating lymphocytes as well as the up-regulation of NFAT2 in each infiltrating lymphocyte.

However, high magnification view showed differential distribution of NFAT2 inside the mucosa infiltrating lymphocytes among UC, CD, and controls. NFAT2 was restricted in the cytoplasm of lymphocytes in normal

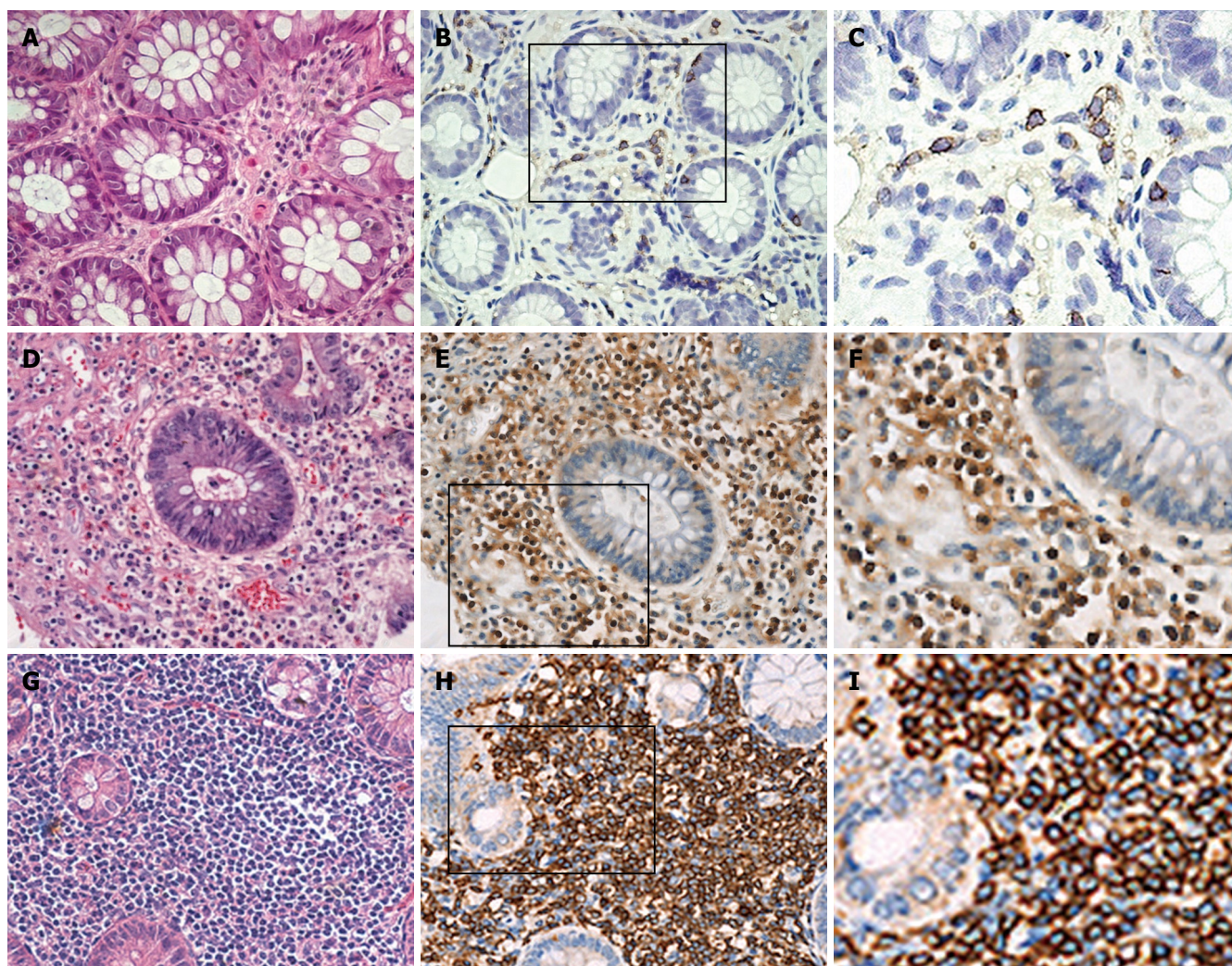


Figure 2 Immunohistochemical analysis of the expression of NFAT2 in colon mucosa tissues. **A-C** are derived from a case of normal control, **D-F** from a case of UC, and **G-I** from a case of CD. **A, D, G** are the results of H&E stain in 100 × magnification. **B, E, H** are the results of immunohistochemistry for NFAT2 counter-staining with hematoxylin for nuclei, 100 × magnification. **C, F, I** are the close view of **B, E, H** respectively. Of note, NFAT2 was exclusively located in cytoplasm of LMPCs of normal colon mucosa as well as LMPCs of CD affected colonic mucosa, whereas NFAT2 was primarily located in the nuclei of LMPCs of the UC affected colonic mucosa.

colonic mucosa (Figure 2B and C), while it was primarily located in the nuclei of infiltrating lymphocytes in UC colonic mucosa (Figure 2E and F). NFAT2 was also restricted in the cytoplasm of infiltrating lymphocytes in CD colon tissue (Figure 2H and I), although there was a heavy infiltration of lymphocytes (Figure 2G-I). Nucleo-translocation of NFAT2 within the infiltrating lymphocytes in UC colonic mucosa, but not in CD affected or normal colonic mucosa, was further validated by confocal microscopy, in which the subcellular distribution of NFAT2 was co-localized with chromosomal DNA within the nuclei of LMPCs in UC colon mucosa (Figure 3).

The cell-types of LMPC with NFAT2 nucleo-translocation/activation in the UC affected colonic mucosa were determined by double-staining using monoclonal antibodies against either CD3, CD4, or CD20, with those against NFAT2. The studies revealed that that nucleo-translocation of NFAT2 primarily occurred in CD8+ T cells and less prominent in CD4+ T cells (data not shown). NFAT2 was also expressed in some of the CD20+ B cells and translocated into nuclei, but mainly localized in the peripheral region of the nuclei (data not shown).

Nucleo-translocation/activation of NFAT2 specifically in UC

To further examine whether nucleotranslocation of NFAT2 of LMPCs in UC, we compared the degree of nucleotranslocation of NFAT2 in LMPCs in UC, DC and non-specific colitis. Diseased colonic tissue sections were obtained from 107 UC cases, 15 CD cases, and 48 cases of non-specific colitis. As shown in Figure 4 and summarized in Table 1, 76%, 26.7% and 8.3% of UC, CD and NC patients had high degree (Grade IV and V) of nuclear translocation of NFAT2, whereas 12%, 60% and 83% of UC, CD and NC patients had low degree (Grade I and II) of nucleotranslocation of NFAT. Obviously, nuclear translocation of NFAT2 within LMPCs was specific for ulcerative colitis ($P < 0.001$ for all three groups, *via* Kruskal-Wallis statistic test; $P < 0.001$ for UC *vs* CD; $P < 0.001$ for UC *vs* NC; $P = 0.023$ for CD *vs* NC, *via* Mann-Whitney test).

Nucleo-translocation/activation of NFAT2 and endoscopy, histologic grading, and clinical outcome

The degree of nucleotranslocation of NFAT2 in LMPCs was correlated to the clinical presentations of the cases

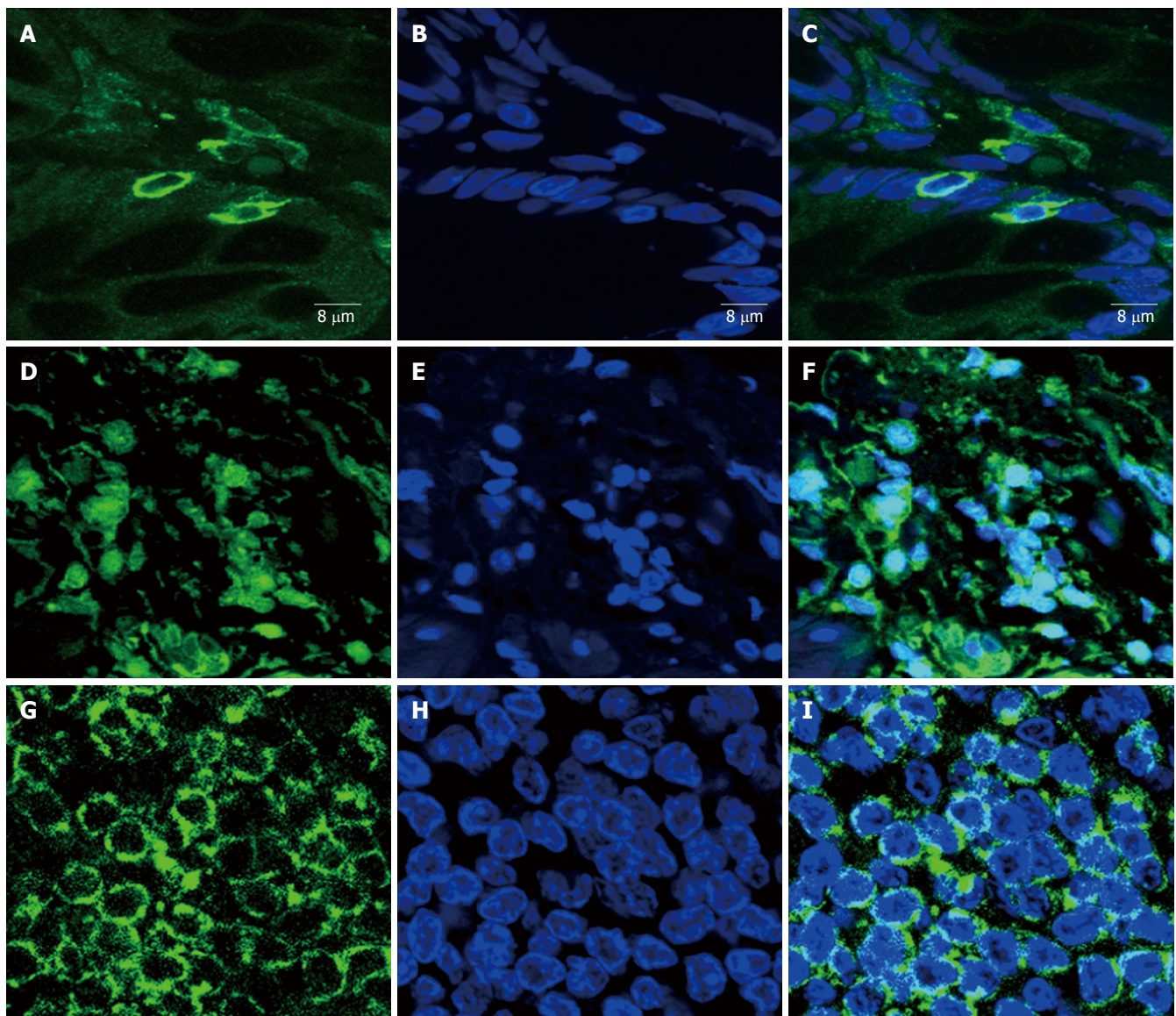


Figure 3 Confocal microscopy demonstrating the subcellular distribution of NFAT2 in LMPCs of colon mucosa. **A-C** are derived from normal colon mucosa, while **D-F** from UC affected mucosa, **G-I** from CD affected mucosa. **A, D, G** represent the detection for NFAT2; **B, E, H**: The nuclei using DAPI; **C, F, I**: the merged images for **A, B, D, E, G** and **H**, respectively. Of interest, co-localization of NFAT2 with nuclei is noted in the colonic mucosa of UC (**F**), but not in CD (**I**), though there is a heavy infiltration of LMPCs in the CD affected colonic mucosa.

of UC, including gender, age at onset, disease duration before sampling, mucosal pattern (endoscopy grading), histologic grading, and outcome (Table 2). The degree of nucleo-translocation/activation of NFAT2 was not related to patients' gender, onset age, and disease duration. It was, however, highly associated with endoscopic grade (correlation coefficient, $\tau = 0.2145$, $P = 0.0281$ *via* Kendall's-rank correlation coefficient), and the histologic grade (correlation coefficient, $\tau = 0.4167$, $P = 0.000$ *via* Kendall's-rank correlation coefficient) and vice versa. There was no significant association between the degree of nucleotranslocation of NFAT2 and long-term clinical outcome of UC including requiring surgical intervention ($P = 0.164$ *via* Fisher's exact test) and survival ($P = 0.081$, *via* Fisher's exact test) (Table 2).

On the other hand, the outcome of long-term follow-up was strongly associated with disease duration before sampling ($P = 0.000$ *via* Kendall's-rank correlation

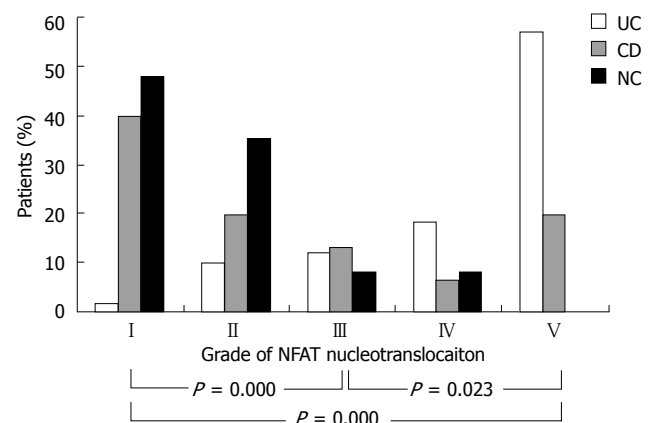


Figure 4 Nucleo-translocation of NFAT2 in UC, CD, and non-specific colitis. Nucleo-translocation of NFAT2 was assayed using immuno-histochemical staining and the tissue sections were obtained from a total of 107 cases of UC, 15 cases of CD and 3 cases of non-specific colitis (NC). Overall P value < 0.001 (determined *via* Kruskal-Wallis statistic).

Table 1 Comparison of the degree of NFAT2 nucleo-translocation in LPMCs in patients of UC, CD and non-specific colitis

Grades of NFAT2 nucleo-translocation in LPMCs (%)	Ulcerative colitis (cases) (%) ¹	Crohn's disease (cases) (%) ¹	Non-specific colitis (cases) (%) ¹
I (0)	2 (01.9)	6 (40.0)	23 (47.9)
II (< 10)	11 (10.3)	3 (20.0)	17 (35.4)
III (10-50)	13 (12.1)	2 (13.3)	4 (8.3)
IV (50-90)	20 (18.7)	1 (06.7)	4 (8.3)
V (> 90)	61 (57.0)	3 (20.0)	0 (0.00)
Total cases	107	15	48

¹*P* = 0.000 amongst UC, CD and NC groups, *via* Kruskal-Wallis statistics; *P* = 0.000 for UC *vs* CD, *via* Mann-Whitney's test; *P* = 0.000 for UC *vs* NC, *via* Mann-Whitney's test; *P* = 0.023 for CD *vs* NC, *via* Mann-Whitney's test.

Table 2 Correlation of NFAT2 nucleotranslocation to clinical presentations

	<i>P</i> value	Statistic methods
Degree of NFAT2 nucleotranslocation ¹		
Onset age (yr, mean ± SD) ¹	0.9484	<i>t</i> -test
Gender (M <i>vs</i> F) ¹	0.651	χ ² test
Disease duration (< 3 m <i>vs</i> > 3 m) ¹	0.507	χ ² test
Outcome (survived <i>vs</i> non-survived) ³	0.081	Fisher's exact test
Outcome (colectomy <i>vs</i> non-colectomy) ¹	0.164	Fisher's exact test
Degree of NFAT2 nucleotranslocation ²		
Endoscopic grade (1-4) ²	0.0281	Kendall's-rank correlation coefficient τ = 0.2145
Histological grade (1-4) ²	0	Kendall's-rank correlation coefficient τ = 0.4167

¹The degree of NFAT2 nucleo-translocation is divided into < 50% *vs* > 50% of LPMCs. ²The degree of NFAT2 nucleo-translocation is categorized into 0%, < 10%, 10%-50%, 50%-90%, > 90% of LPMCs. ³Non-survived: Died of complications of UC.

coefficient), endoscopy grade (*P* = 0.000 *via* Kendall's-rank correlation coefficient) and histologic grade (*P* = 0.031 Kendall's-rank correlation coefficient), but not associated with the degree of nucleo-translocation/activation of NFAT2 (*P* = 0.185 *via* Kendall's-rank correlation coefficient), age (*P* = 0.406), or sex (*P* = 0.369).

DISCUSSION

Though many studies and reports have addressed the role of cytokines in the development of IBD, there is still a great gap between the increase of the putative initiating cytokines, such as IL-4 and IFN-γ (around 3 fold), and the downstream profound cascade of pro-inflammatory cytokines that include TNF-α, IL-1β and IL-6 (around 10-20 folds), which are directly implicated in the inflammatory process^[10-12,33,34]. Indeed, the mechanisms that multiply downstream signaling and cause intractable inflammatory events remains completely unclear.

We report here the nucleo-translocation/activation of NFAT2 of lamina propria mononuclear cells (LPMCs) in UC affected colonic mucosa. The nucleo-translocation/activation of NFAT2 of LPMCs is specific for UC

because it is relatively rare in CD and is rarely found in non-specific colitis. Of interest, it has been shown that the activation of NFAT2 primarily leads to the commitment of Th2 differentiation^[35-37]. Our findings of the nucleo-translocation/activation of NFAT2 specifically in UC is consistent with the general notion that UC is primarily driven by a Th2-like immune activation that is different from the major roles of Th1 immune activation in CD^[10-14]. In addition, we show that the degree of nucleo-translocation/activation of NFAT2 of LPMC has been strongly associated with disease activity, such as clinical and histological grading, indicating its direct implication in the pathogenesis of UC. Our findings are reminiscent of that reported by Neurath *et al*, in which activation of T-bet, a transcription factor directing Th1 cell development and regulating T cell function, in the LPMCs was specifically found in CD patients but not in UC patients^[38]. In addition, over-expression of T-bet was found to be essential and sufficient to promote Th1-mediated colitis in an experimental mouse model of CD^[38].

NFAT proteins, which are originally identified in T cells as inducers of cytokine gene expression, play a variety of biological roles in the differentiation of many cell types, including effector T cells, osteoclasts, and muscle cells^[36,37,39-41]. The NFAT family contains five transcription factors, four of which (NFAT1-4) are regulated by the calcium/calcineurin signaling pathways that are essential for the regulation of lymphocyte function. The only non-calcium-regulated NFAT protein, NFAT5, is expressed in response to osmotic stress by almost all cells^[40,42].

Activation of NFAT1-4 proteins is tightly regulated by calcium-dependent phosphatase calcineurin. Calcineurin signaling was first defined in T lymphocytes as a regulator of nucleo-translocation and activation of NFAT proteins. In resting cells, NFAT proteins reside in the cytoplasm and are heavily phosphorylated. Upon engagement of cell surface receptors, the membrane component phosphatidylinositol-4,5-bisphosphate is converted to inositol-1,4,5-trisphosphate (IP3) and diacylglycerol. IP3 sequentially induces the calcium release from the intracellular store and triggers the opening of calcium-release-activated calcium channels in cytoplasm membrane, thereby maintaining the increased levels of intracellular calcium. This, in turn, forms a complex with calmodulin that activates the Ser/Thr-phosphatase activity of calcineurin so as to de-phosphorylate NFAT proteins resulting in nucleo-translocation of NFAT proteins and the induction of NFAT-dependent gene transcription^[40,43,44].

NFAT1 and NFAT2 are the major NFAT proteins in peripheral T cells; they overlap in function but differ remarkably in mode of expression. NFAT1 is constitutively synthesized in T cells, whereas NFAT2, the most prominent NFAT proteins in peripheral T cells, is expressed upon following T-cell receptor and co-receptor stimulation and maintained by an auto-regulation mechanism^[40,45]. Activation of NFAT proteins, together with other transcription factors (such as T-bet, STAT1, 4, 6, GATA3, and FOXP3), determines the TH1/TH2/Treg lineage choice, particularly the development of TH2 immune response that is dependent on the nature of the stimulus and the signals that are received

from specific cytokines^[34,40,45,46]. Upon the engagement of TCR, for example, NFAT2 cooperates with MAT to induce the expression of IL-4, IL-5 and TH2 lineage specific transcription factors (such as NFAT2, STAT6 and GATA3) to commit to TH2 cell differentiation^[36,37,40,47-49].

Of interest, there are two NFAT binding sites in each of the two promoter regions of the NFAT2 gene. Mutations abolishing NFAT binding result in a profound decrease in the transcription of NFAT2. Obviously, there is a positive auto-regulation of NFAT2 expression in TCR activated T cells^[43,50,51]. Taken together, nucleo-translocation/activation of NFAT2 inflames an auto-regulatory positive feedback loop in amplifying downstream pro-inflammatory signaling. Our findings of specific nucleo-translocation/activation of NFAT2 in LPMCs in UC might imply the answer to the long term enigma of the mechanisms leading to the ultimate cascade of intractable inflammatory process of UC^[52].

The strong association of aberrant nucleotranslocation/activation of NFAT2 in LPMCs with the disease activity of UC further supports our hypothesis of direct implication of the calcium-calcieneurin/NFAT pathways in the pathogenesis of UC. Blocking the positive-feedback loops initiated by the activation of NFAT2 would not only achieve an instant anti-inflammatory effect but might also lead to a long term remission of the disease. The calcium-calcieneurin/NFAT pathways can therefore be the therapeutic targets for UC treatment. Indeed, targeting the calcium-calcieneurin/NFAT pathways in the treatment of other chronic immune-mediated and intractable auto-immune diseases has been well documented^[53]. For the treatment of UC, cyclosporin A, a specific inhibitor of calcieneurin activity, has also been reported to be a successful treatment in cases with severe or steroids intractable UC^[22,54-59]. Cyclosporin A has also been suggested as the consensus treatment for patients with severe UC in whom standard therapy has failed, or for those who are candidates for colectomy^[60]. Nevertheless, the clinical use of the potent immunosuppressants, such as cyclosporin A (CsA) and tacrolimus (FK506), the fungal metabolites blocking the phosphatase activity of calcieneurin, and consequently inhibiting the de-phosphorylation and nucleo-translocation of NFAT proteins, can cause severe side effects and, as such, their prolonged use in many chronic inflammatory or autoimmune diseases has been restricted. Recently, there has been a merging of peptide sequences that more specifically interfere with the interaction between calcieneurin and NFAT, as well as small organic molecules that modulate NFAT function as the potential new immunosuppressive drug with improved specificity and reduced toxicity. Application of similarly newly developed drugs to the treatment of UC and some of CD will open the new era of IBD therapy in the near future^[22-29,61-63].

In summary, since the activation of NFAT is implicated in an auto-regulatory positive feedback loop of sustained T-cell activation, our findings of specific nucleo-translocation/activation of NFAT2 in LPMCs of UC not only indicate the pivotal roles of activation of NFAT2 pathways in the intractable inflammatory process of UC for the first time, but also open a window for the future development of new strategies for UC therapy.

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COMMENTS

Background

We used two-dimension gel electrophoresis comparing the proteome difference between the ulcerative colitis (UC). Affected and unaffected colonic mucosa. Of the up-regulated proteins in the UC affected colonic mucosa, nuclear factor of activated T cell 2 (NFAT2) was particularly interesting, since NFAT2 is not only the major NFAT proteins in peripheral T cells but also playing key roles in regulating T cell differentiation and functions. We aimed to investigate the role of NFAT2 in the development of UC.

Research frontiers

Although T cells play pivotal roles in the pathogenesis of ulcerative colitis, the mechanisms causing sustained T cell activation and intractable inflammation remain unknown. It has been shown that activation of NFAT2 is implicated in an auto-regulatory positive feedback loop leading to a sustained T-cell activation. However, the role of NFAT in the pathogenesis of UC has never been addressed.

Innovations and breakthroughs

This is the first study addressing the strong association of nucleo-translocation/activation of NFAT2 of the lamina propria lymphocytes in the UC affected mucosa with the disease activity and severity. More interestingly, nucleo-translocation/activation of NFAT2 of the lamina propria lymphocytes in the colonic mucosa was specific for UC, since it was less prominent in Crohn's disease (CD) and rarely detected in non-specific colitis, suggesting the roles of NFAT2 activation in the pathogenesis of UC.

Applications

Because NFAT proteins are regulated by the calcium/calcieneurin signaling pathways, our findings suggest direct implication of the calcium/calcieneurin signaling pathways in the pathogenesis of UC. Blocking the positive-feedback loops initiated by the activation of NFAT2 would not only achieve an instant anti-inflammatory effect but might also lead to a long term remission of the disease. The calcium-calcieneurin/NFAT pathways can therefore be the therapeutic targets for UC treatment.

Terminology

NFAT proteins originally identified in T cells as inducers of cytokine gene expression play a variety of biological roles in the differentiation of many cell types, including effector T cells, osteoclasts, and muscle cells. NFAT2 is the most prominent NFAT proteins in peripheral T cells. Activation of NFAT2 determines the TH1/ TH2/Treg lineage choice, particularly the development of TH2 immune response.

Peer review

In this study, the authors demonstrated for the first time the strong association of activation of nuclear factor of activated T lymphocytes with the disease activity and severity of UC. This work adds significant information regarding the mechanisms of sustained T cell activation and intractable inflammation of UC, and opens a new window for future development of anti-UC therapy.

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CLINICAL RESEARCH

Endoscopic and histopathological study on the duodenum of *Strongyloides stercoralis* hyperinfection

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Abstract

AIM: To investigate endoscopic and histopathological findings in the duodenum of patients with *Strongyloides stercoralis* (*S. stercoralis*) hyperinfection.

METHODS: Over a period of 23 years (1984-2006), we investigated 25 patients with *S. stercoralis* hyperinfection who had had an esophagogastroduodenoscopy before undergoing treatment for strongyloidiasis. The clinical and endoscopic findings were analyzed retrospectively.

RESULTS: Twenty-four (96%) of the patients investigated were under immunocompromised condition which was mainly due to a human T lymphotropic virus type 1 (HTLV-1) infection. The abnormal endoscopic findings, mainly edematous mucosa, white villi and erythematous mucosa, were observed in 23 (92%) patients. The degree of duodenitis including villous atrophy/destruction and inflammatory cell infiltration corresponded to the severity of the endoscopic findings. The histopathologic yield for identifying larvae was 71.4% by duodenal biopsy. The endoscopic findings of duodenitis were more severe in patients whose biopsies were positive for larvae than those whose biopsies were negative (Endoscopic severity score: 4.86 ± 2.47 vs 2.71 ± 1.38 , $P < 0.05$).

CONCLUSION: Our study clearly demonstrates that, in addition to stool analysis, endoscopic observation and biopsies are very important. We also emphasize that *S. stercoralis* and HTLV-1 infections should be ruled out before immunosuppressive therapy is administered in endemic regions.

INTRODUCTION

Strongyloides stercoralis (*S. stercoralis*) is an intestinal nematode which is a parasite of humans. About 100 million people are infected by this parasite in tropical and subtropical areas^[1]. Infections are acquired when larvae penetrate the skin and migrate to the duodenum and upper jejunum to mature. An internal autoinfective cycle allows the parasite to reside within a human for years. Clinical syndromes of *S. stercoralis* vary widely. Chronic infection with *S. stercoralis* is most often asymptomatic. Hyperinfection describes a syndrome of accelerated autoinfection which results from immunosuppression. Detection of an increased number of larvae in stool, sputum and/or tissue is a hallmark of hyperinfection^[2]. Gastrointestinal and pulmonary symptoms are common but non-specific, and include abdominal pain, diarrhea, vomiting, adynamic ileus, small bowel obstruction (SBO) and protein-losing enteropathy, as well as pneumonia. Disseminated infection is the migration of larvae to organs beyond the range of the autoinfective cycle (lungs and gastrointestinal tract) and is often complicated by Gram-negative sepsis. Such organs include the skin, liver, central nervous system as well as virtually every other organ. An immunocompromised condition consists of immunosuppressive drug therapy (e.g., corticosteroids, cyclosporine and anti-cancer drugs), hematologic malignancies, organ transplants, human T lymphotropic virus type 1 (HTLV-1) infection, and human immunodeficiency virus (HIV) infection^[1,2]. As *S. stercoralis* colonizes in the duodenum where the larvae

mature, endoscopic evaluation has been recognized as an important tool for diagnosing strongyloidiasis. Although there have been several reports demonstrating endoscopic findings of strongyloidiasis, most of these are case reports or small case series^[3-19]. This study aims at investigating the relationship of endoscopic markers to clinical and histopathological findings of the duodenum in patients with *S. stercoralis* hyperinfection in an endemic region.

MATERIALS AND METHODS

Patients

Over a 23-year period from 1984 to 2006, we identified 25 patients (15 males and 10 females; mean age = 63.0 ± 14.1 years) with *S. stercoralis* hyperinfection who had had an esophagogastroduodenoscopy (EGD) before undergoing treatment for strongyloidiasis at Ryukyu University Hospital and other affiliated hospitals in Okinawa, Japan. The diagnosis of *S. stercoralis* hyperinfection was based on gastrointestinal, pulmonary and/or systemic symptoms along with the identification of an increased number of *Strongyloides* larvae or ova in the stool, sputum, gastroduodenal drainage and/or tissue. The clinical and endoscopic findings of the patients were investigated retrospectively. The study was conducted and carried out in accordance with the Helsinki Declaration.

Endoscopy and histopathology

EGD was performed with forward-viewing endoscopes (Olympus, Tokyo, Japan) at the second portion of the duodenum. Since the endoscopic severity scoring system of the duodenum has not been established, we created the scoring system, which was determined by the total number of points for duodenitis. One point was given for a mild form (edema, erythema and white villi), two points for a moderate form (erosion, fine granule and hemorrhage) and three points for a severe form (ulcer, dilatation, dilatation and pseudopolyps). At endoscopy, biopsy specimens were obtained from the duodenal mucosa in a routine fashion using standard forceps. The specimens were stained with hematoxylin and eosin for histopathological evaluation. Duodenal pathology was assessed as described elsewhere^[20].

Statistical analysis

Mann-Whitney *U*-test was used, when appropriate, to compare any differences among the groups. Statistical comparisons were analyzed using SPSS for Windows version 15 (SPSS Inc., Japan). *P* values less than 0.05 were considered statistically significant.

RESULTS

Clinical features

The clinical features of the patients are summarized in Table 1. The main symptoms and/or illnesses complicated by the hyperinfection were as follows: vomiting in 13 (52.0%), abdominal pain in 10 (40.0%), diarrhea in 8 (32.0%), SBO in 8 (32.0%), weight loss in 7 (28.0%), bacterial meningitis in 3 (12.0%), sepsis in 3 (12.0%), pneumonia in 2 (8.0%), and gastrointestinal bleeding in 2

(8.0%) patients. Most patients had more than one clinical symptom or illness. Six patients had disseminated infection (bacterial meningitis and/or sepsis) and the outcome of 2 patients was fatal. Twenty-four (96%) patients were under immunocompromised conditions resulting from HTLV-1 infection, administration of corticosteroid, diabetes mellitus, alcoholism, liver cirrhosis and chronic renal failure. Of note, 18 (72.0%) patients were HTLV-1 carriers. The diagnostic samples used for identifying *Strongyloides* were as follows: stool samples from 17 (68.0%) patients, duodenal biopsy from 15 (60.0%) patients, gastroduodenal drainage from 9 (36.0%) patients and sputum from 3 (12.0%) patients. Most patients were treated with thiabendazole or ivermectin alone and 2 patients needed a combination of the two drugs.

Endoscopic and histopathological findings

The endoscopic findings of the patients are summarized in Table 2. Gross abnormal findings were observed in 23 (92.0%) patients and normal findings in 2 (8.0%) patients. A broad range of endoscopic findings included edematous mucosa in 16 (69.5%) patients, white villi in 13 (56.5%), erythematous mucosa in 9 (39.1%), erosion in 6 (26.0%), stenosis in 4 (17.3%), fine granule in 4 (17.3%), hemorrhage in 3 (13.0%), dilatation in 3 (13.0%), and ulcer in 2 (8.6%) patients (Figure 1). Most patients had more than one finding. Strongyloidiasis was diagnosed histopathologically in 71.4% (15/21) of patients who had duodenal biopsies. According to the endoscopic severity score of the duodenum, the endoscopic findings of duodenitis were more severe in patients whose biopsies were positive for larvae than in those with negative biopsies (4.86 ± 2.47 vs 2.71 ± 1.38 , $P < 0.05$, Figure 2). Representative endoscopic images of white villi and edematous mucosa (Figure 3), white villi and stenosis (Figure 4), and ulcer and pseudopolyps (Figure 5) are shown along with the histopathological findings. As shown together with the endoscopic and histopathological images, the degree of duodenitis, including villous atrophy/destruction and inflammatory cell infiltration, was associated with the severity of the endoscopic findings. In 6 cases whose stool was not obtained due to SBO or larvae were not identified in the stool, strongyloidiasis was diagnosed only by duodenal biopsies.

DISCUSSION

This study was conducted in the Okinawa islands, a subtropical region of Japan, where both *S. stercoralis* and HTLV-1 are endemic and epidemiological studies have been thoroughly conducted^[21-24]. There is an increasing body of evidence regarding the strong association between *S. stercoralis* and HTLV-1 co-infection and hyperinfection syndrome^[21-26], which has been further strengthened by our striking result that a majority (72%) of the patients with hyperinfection were co-infected with HTLV-1. We also confirmed the well-described role of corticosteroids in triggering hyperinfection regardless of the presence of HTLV-1 co-infection. Corticosteroids not only have a well-known effect impairing human immunity but also directly affect the female larvae to increase output of infective

Table 1 Clinical characteristics of patients with *S. stercoralis* hyperinfection

Case No.	Age/Gender	Presenting symptoms and/or illness	Immunosuppressive state	Diagnosis	Treatment	Outcome
1	51/M	Meningitis, GI bleeding	Alcoholism	Duodenal biopsy, stool	TBZ	Cured
2	72/M	Diarrhea	HTLV-1	Stool	TBZ	Cured
3	80/M	Sepsis, meningitis	HTLV-1, corticosteroids	Duodenal biopsy, stool, sputum, gastroduodenal drainage	TBZ	Cured
4	38/M	Abdominal pain	HTLV-1	Duodenal biopsy, stool	TBZ	Cured
5	58/F	Sepsis, diarrhea, pneumonia	Corticosteroids	Sputum	IVM	Dead
6	86/F	Vomiting, weight loss	HTLV-1, DM	Duodenal biopsy, stool, sputum	IVM	Cured
7	31/F	SBO, abdominal pain, vomiting	HTLV-1, alcoholism	Duodenal biopsy, stool, gastroduodenal drainage	TBZ	Cured
8	58/F	Vomiting, weight loss	HTLV-1	Duodenal biopsy, gastroduodenal drainage	IVM	Cured
9	62/M	Meningitis, diarrhea	HTLV-1, DM	Duodenal biopsy, gastroduodenal drainage	TBZ	Cured
10	73/F	Abdominal pain	HTLV-1, liver cirrhosis	Stool	IVM	Cured
11	58/M	SBO, abdominal pain, vomiting	HTLV-1, DM	Duodenal biopsy	IVM, TBZ	Cured
12	52/M	SBO, abdominal pain, vomiting	HTLV-1	Duodenal biopsy, stool, gastroduodenal drainage	TBZ	Cured
13	74/M	Diarrhea, weight loss	Chronic renal failure	Stool	IVM	Cured
14	66/F	Vomiting, diarrhea	HTLV-1	Duodenal biopsy, stool	TBZ	Cured
15	42/M	SBO, abdominal pain, GI bleeding	HTLV-1	Duodenal biopsy, stool	IVM, TBZ	Cured
16	56/F	Vomiting, abdominal pain	HTLV-1	Duodenal biopsy, gastroduodenal drainage	TBZ	Cured
17	62/M	SBO, abdominal pain, vomiting	None	Stool	TBZ	Cured
18	80/M	SBO, abdominal pain, vomiting	Alcoholism	Stool	IVM	Cured
19	82/M	SBO, meningitis, pneumonia	DM	Stool, sputum	IVM	Dead
20	57/M	Diarrhea, weight loss	HTLV-1	Stool	IVM	Cured
21	76/F	Vomiting, weight loss	HTLV-1	Duodenal biopsy, stool	TBZ	Cured
22	50/F	SBO, abdominal pain, vomiting	HTLV-1, corticosteroids	Duodenal biopsy	IVM	Cured
23	66/M	Vomiting, weight loss	HTLV-1	Duodenal biopsy, gastroduodenal drainage	IVM	Cured
24	71/M	Sepsis, diarrhea	Corticosteroids	Gastroduodenal drainage	IVM	Cured
25	74/F	Vomiting, diarrhea, weight loss	HTLV-1	Gastroduodenal drainage, stool	IVM	Cured

GI: Gastrointestinal; SBO: Small bowel obstruction; DM: Diabetes mellitus; TBZ: Thiabendazole; IVM: Ivermectin.

larvae with a structural similarity to larval ecdysteroids^[1,27].

Enteritis by *S. stercoralis* has been studied since the early 1960's before the endoscopic era. de Paola *et al*^[28] classified the histopathological changes in fatal cases into three forms: catarrhal enteritis, edematous enteritis and ulcerative enteritis. Catarrhal enteritis is a minor form characterized by mild mucosal congestion with larvae restricted to the mucous membrane. Edematous enteritis is a moderately serious form characterized by edematous thickening of the wall, swelling folds and villous atrophy. Larvae occupy lymph-vessel spaces. They also observed that mucosal edema was not only a result of inflammation and protein deficiency but also an effect of larval invasion in the lymph vessels and lymphangiectasia. Ulcerative enteritis is a serious form characterized by ulcers and fibrosis. Larvae are encountered in the entire wall. In the endoscopic era, there have been several case reports describing endoscopic findings of the duodenum in strongyloidiasis, including normal mucosa, edema, erythema, erosion, swollen folds, fine granule, tiny ulcer, polyps, hemorrhage, megaduodenum, deformity, and stenosis (Table 3). To our knowledge, the present retrospective study represents the largest endoscopic experience with *S. stercoralis* hyperinfection. In the histopathological studies, Coutinho *et al*^[29] reported that duodenal villous atrophy and crypt hyperplasia were proportional to the degree of clinical severity of strongyloidiasis. Suarez and Sanchez^[19] confirmed that plasma cell infiltration, villous atrophy

and severe duodenitis were the characteristics of severe strongyloidiasis. A recent study clearly demonstrated that strongyloidiasis disrupted epithelial kinetics in the human small intestine by the induction of apoptosis and inhibition of cell proliferation, thereby resulting in villous atrophy and impaired barrier function^[17]. Our observations disclosed that endoscopic findings of duodenitis were more severe in the patients whose biopsies were positive for larvae than in those with negative biopsies. Considering the fact that increased numbers of larvae are strongly associated with the progression to hyperinfection, our result supports their findings.

Prior reports have indicated that findings frequently include edematous mucosa, swollen folds and erythematous mucosa. However, pathognomonic findings are apparently not evident^[3-19]. Our present study confirmed the aforementioned frequent findings. In addition, we noticed that an endoscopic feature of duodenal white villi seemed to be a frequent finding. The findings of tiny white spots^[9] and white punctuate dotting mucosa^[11] appear to be similar in appearance. The endoscopic finding of white villi is well-known in intestinal lymphangiectasia with protein-losing enteropathy. It represented markedly dilated lymphatics in the stroma of the villi and fats, including fat droplets in the absorptive cells from the impaired transport of fats from intestinal epithelial cells to intestinal lymphatics^[30,31]. Considering the fact that larvae invade the lymph vessels and that there

Table 2 Endoscopic and histopathological findings of the duodenum

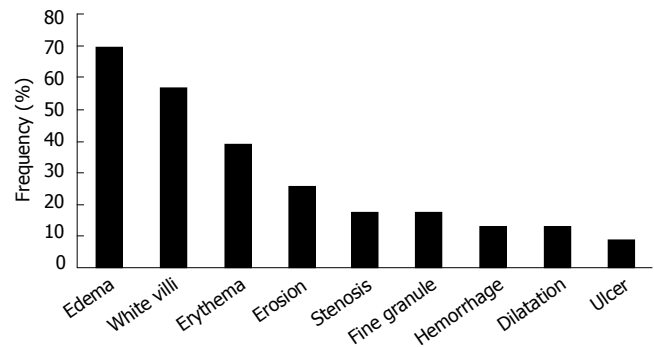
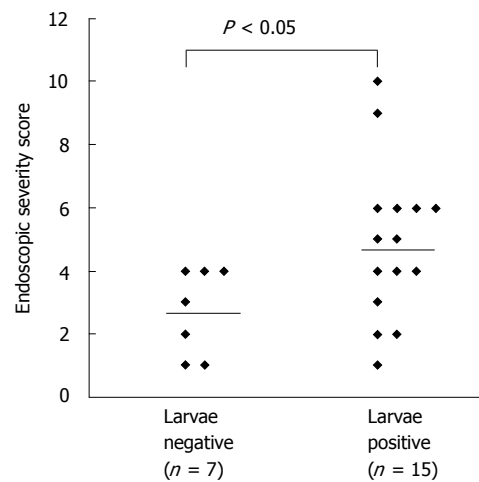
Case No.	Endoscopic findings of the duodenum	Histopathologic detection of <i>S. stercoralis</i> in the duodenum
1	Edema, erythema, erosion, hemorrhage	Larvae
2	Erythema	Negative
3	Edema, white villi, erosion	Larvae
4	Edema, white villi, erythema, dilatation	Larvae
5	White villi	ND
6	Edema, fine granule	Larvae
7	Erythema	Larvae
8	Edema, erosion, stenosis	Larvae
9	Edema, white villi	Larvae
10	Normal	ND
11	Edema, white villi, dilatation	Larvae
12	Edema, white villi	Larvae
13	White villi, erythema	Negative
14	Edema, white villi, fine granule	Larvae
15	Edema, erythema, ulcer, hemorrhage, pseudopolyps	Larvae
16	Edema, white villi, stenosis	Larvae
17	Edema, stenosis	Negative
18	White villi, erythema, erosion	Negative
19	Edema, ulcer	Negative
20	Normal	ND
21	Edema, white villi, erosion, hemorrhage	Larvae
22	Edema, white villi, erosion, fine granule, stenosis	Larvae
23	Fine granule, dilatation	Larvae
24	Edema, white villi, erythema	Negative
25	Erythema	Negative

Edema: Edematous mucosa; Erythema: Erythematous mucosa; ND: Biopsies were not done.

is subsequent lymphangiectasia in edematous enteritis as reported by de Paola *et al*^[28], the appearance of white villi may reflect villous atrophy/destruction and mucosal edema similar to intestinal lymphangiectasia. We, therefore, emphasize that white villi can be a good endoscopic marker for strongyloidiasis in endemic regions.

There have been very few reports regarding the histopathologic yield by endoscopy for strongyloidiasis. In a study by Thompson *et al*^[13], a minimum of six biopsies were obtained from each lesion, resulting in a 100% histopathologic yield from the 6 patients. The reason for our low yield (71.4%) may be due to the fact that our study is a retrospective study conducted at multiple hospitals and only one to three biopsies were taken from each lesion. Obtaining multiple biopsy specimens might increase the histopathologic yield. However, looking at our study from another point of view, only duodenal biopsies were able to establish a diagnosis when the stool analysis was negative in 6 (24%) patients, which lead to the avoidance of a fatal outcome.

In conclusion, *S. stercoralis* hyperinfection can rapidly become fatal, so early diagnosis and treatment is very important. Although diagnosis is usually made by stool analysis, our results clearly demonstrate that endoscopic observation and biopsies, in addition to gastroduodenal drainage analysis, are important tools for diagnosing strongyloidiasis. We also emphasize that infection with *S. stercoralis* and HTLV-1 should be ruled out before

**Figure 1** Frequency of abnormal endoscopic findings in the duodenum**Figure 2** Comparison of endoscopic severity of the duodenum between the patients with larvae present and larvae absent in the duodenal biopsy ($P < 0.05$, Mann-Whitney U-test).

immunosuppressive therapy is administered for patients living in or coming from endemic regions.

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COMMENTS

Background

Strongyloides stercoralis (*S. stercoralis*) is an intestinal nematode that infects about 100 million people worldwide. As *S. stercoralis* colonizes in the duodenum, endoscopic evaluation has been recognized as an important tool for diagnosing strongyloidiasis. Although there have been several case reports demonstrating endoscopic findings of strongyloidiasis, the relationship of endoscopic markers to clinical and histopathological findings have not been intensively studied.

Research frontiers

There is an increasing body of epidemiological evidence regarding the association between *S. stercoralis* infection and HTLV-1 infection. Studies of molecular mechanism of this association have become one of the hot spots at present. For the diagnostic purpose, detection of *S. stercoralis* in clinical samples has been improved by the agar plate culture method which was invented recently.

Related publications

Nakada *et al*^[21] clearly disclosed the first evidence of the association between *S. stercoralis* infection and HTLV-1 infection. Hirata *et al*^[24] conducted a large

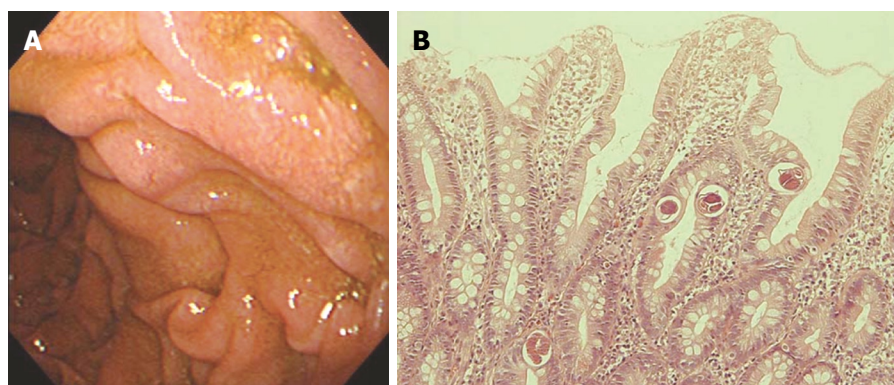


Figure 3 Representative endoscopic image and HE staining of duodenal biopsy. **A:** EGD showing white villi and edematous mucosa in the second part of duodenal (Case 11); **B:** Biopsy specimen from the mucosa showing numerous larvae with villous atrophy and mild inflammatory cell infiltration (HE, $\times 200$).

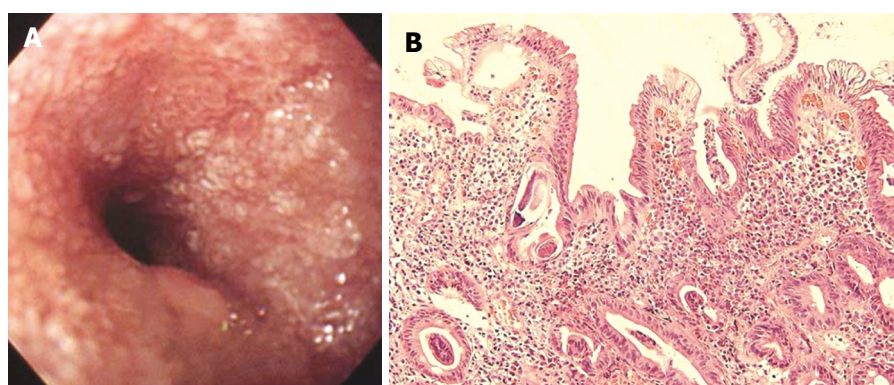


Figure 4 Endoscopic image and HE staining of duodenal biopsy. **A:** EGD showing white villi and stenosis in the second part of duodenal (Case 16); **B:** Biopsy specimen from the mucosa showing numerous larvae with severe villous atrophy and moderate inflammatory cell infiltration (HE, $\times 200$).

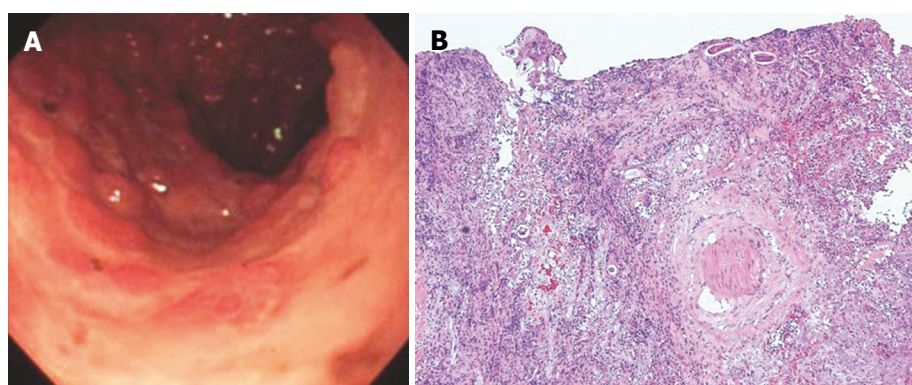


Figure 5 Endoscopic findings and HE staining of duodenal biopsy. **A:** EGD showing large ulcers and pseudopolyps in the second part of duodenal (Case 15); **B:** Biopsy specimen from the margin of the ulcer showing formation of granulation tissue and complete destruction of the villi. Numerous larvae are observed within the granulation and lymph vessels (HE, $\times 100$).

Table 3 Reported endoscopic findings of the duodenum with strongyloidiasis in literature

Authors	Year	No. of cases	Immunosuppressive state	Endoscopic findings of the duodenum
Brasitus <i>et al</i> ^[3]	1980	1	None	Brisk bleeding, deformed bulb
Milder <i>et al</i> ^[4]	1981	2	ND	Enlarged folds
Bone <i>et al</i> ^[5]	1982	1	ND	Deformed cap, obliterated second part
Bhatt <i>et al</i> ^[6]	1990	1	None	Mild erythema
Chen <i>et al</i> ^[7]	1994	1	Corticosteroids	Flattened folds, swelling mucosa, tiny ulcer
Choudhry <i>et al</i> ^[8]	1995	3	DM, none	Multiple serpiginous lesions, duodenal nodule
Hizawa <i>et al</i> ^[9]	1996	1	HTLV-1 and corticosteroids	Edema, tiny white spots
Friedenberg <i>et al</i> ^[10]	1999	1	HTLV-1	Severe stenosis
Overstreet <i>et al</i> ^[11]	2003	1	HIV	White punctate dotting mucosa
Asano <i>et al</i> ^[12]	2004	1	HTLV-1	Fine granule, coarse mucosa, disappeared folds
Thompson <i>et al</i> ^[13]	2004	6	Corticosteroids, DM, HIV, none	Edema, brown discoloration, erythematous spots, subepithelial hemorrhage, megaduodenum
Seet <i>et al</i> ^[14]	2005	1	Anti-myeloma drugs	Erythematous and granular mucosa
Karmo <i>et al</i> ^[15]	2006	1	Corticosteroids	Normal
Ghoshal <i>et al</i> ^[16]	2006	1	Corticosteroids	Multiple nodules
Werneck-Silva <i>et al</i> ^[17]	2006	4	ND	Erythema, erosion
Csermely <i>et al</i> ^[18]	2006	1	Anti-myeloma drugs	Necrotic ulcerations
Suarez and Sanchez ^[19]	2006	11	ND	Swollen folds of nodular aspect

ND: Not determined.

scale epidemiological study which strengthened this association and showed the impairment of host immune response against *S. stercoralis* by HTLV-1 infection.

Innovations and breakthroughs

This study clarified the strong association between *S. stercoralis* hyperinfection and HTLV-1 infection. Moreover, presence of white villi can be a good endoscopic marker for the duodenal strongyloidiasis. Endoscopic biopsy helped early diagnosis of strongyloidiasis.

Applications

Early endoscopic diagnosis of strongyloidiasis can have a marked impact on disease outcome. Co-infection with *S. stercoralis* and HTLV-1 should be ruled out to prevent hyperinfection before immunosuppressive therapy (e.g., corticosteroids) is administered for patients living in or coming from endemic regions.

Terminology

Hyperinfection is a syndrome of accelerated larval autoinfection which results from immunosuppression. Disseminated strongyloidiasis is the migration of larvae to organs beyond the range of the autoinfective cycle and is often complicated by Gram-negative sepsis which results in high mortality rates.

Peer review

This is a well conducted study clarifying the strong association between *S. stercoralis* hyperinfection and HTLV-1 infection.

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RAPID COMMUNICATION

Model for end-stage liver disease score *versus* Child score in predicting the outcome of surgical procedures in patients with cirrhosis

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Abstract

AIM: To determine factors affecting the outcome of patients with cirrhosis undergoing surgery and to compare the capacities of the Child-Turcotte-Pugh (CTP) and model for end-stage liver disease (MELD) score to predict that outcome.

METHODS: We reviewed the charts of 195 patients with cirrhosis who underwent surgery at two teaching hospitals over a five-year period. The combined endpoint of death or hepatic decompensation was considered to be the primary endpoint.

RESULTS: Patients who reached the endpoint had a higher MELD score, a higher CTP score and were more likely to have undergone an urgent procedure. Among patients undergoing elective surgical procedures, no statistically significant difference was noted in the mean MELD (12.8 ± 3.9 vs 12.6 ± 4.7 , $P = 0.9$) or in the mean CTP (7.6 ± 1.2 vs 7.7 ± 1.7 , $P = 0.8$) between patients who reached the endpoint and those who did not. Both mean scores were higher in the patients reaching the endpoint in the case of urgent procedures (MELD: 22.4 ± 8.7 vs 15.2 ± 6.4 , $P = 0.0007$; CTP: 9.9 ± 1.8 vs 8.5 ± 1.8 , $P = 0.008$). The performances of the MELD and CTP scores in predicting the outcome of urgent surgery were only fair, without a significant difference between them (AUC = 0.755 ± 0.066 for MELD vs AUC = 0.696 ± 0.070 for CTP, $P = 0.3$).

CONCLUSION: The CTP and MELD scores performed

equally, but only fairly in predicting the outcome of urgent surgical procedures. Larger studies are needed to better define the factors capable of predicting the outcome of elective surgical procedures in patients with cirrhosis.

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Key words: Liver cirrhosis; Prognosis; Severity of illness index; Surgical procedures; Operative; Postoperative complications

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INTRODUCTION

Patients with cirrhosis who undergo surgery under general anesthesia are at an increased risk of surgery and anesthesia-related complications^[1]. The underlying liver dysfunction makes patients with cirrhosis particularly susceptible to bleeding and infection, two common post-surgical problems^[2]. Moreover, the effect of surgery and anesthesia on the liver may be significant. Whereas the exact hemodynamic effect of different anesthetics on the hepatic circulation can be variable^[3-5], the net result is usually a reduction in hepatic perfusion^[6], thereby exposing the liver to the risk of ischemic injury. In addition, a number of anesthetic agents have a potential for direct, drug-induced hepatotoxicity^[7-9]. Blood loss during surgery may result in hepatic hypoperfusion as well. Post-operative hypotension, sepsis and the subsequent use of medication with potential hepatotoxicity further increase the risk of liver injury^[10]. A number of case series of patients with cirrhosis undergoing specific surgical procedures suggest a worse than expected outcome^[11-15]. A review of

733 patients with cirrhosis undergoing various surgical procedures revealed a perioperative mortality rate of 11.6% and a postoperative complication rate of 30%, which is significantly higher than what would be expected in patients without liver disease^[16].

The significant perioperative risks associated with surgery in patients with cirrhosis highlight the importance of preoperative assessment and appropriate patient selection for surgery. The Child-Turcotte-Pugh (CTP or "Child") classification was initially designed to evaluate the risk of surgical portosystemic shunt procedures, and was subsequently found to predict long-term survival in patients with cirrhosis^[17]. Interestingly, the more recently devised prognostic scoring system used in patients with cirrhosis, namely the Model for End-stage Liver Disease (MELD) score was also originally designed for the purpose of selection of cirrhotic patients for a portosystemic shunt procedure-the transjugular intrahepatic portosystemic shunt (TIPS)^[18]. Whereas the CTP class has traditionally been used in preoperative risk stratification in cirrhotic patients undergoing surgery, the MELD score was found to be superior to the CTP score and class in predicting three-month survival in patients with cirrhosis^[19].

There has been a recent interest in evaluating the role of the MELD score in preoperative risk assessment, with a number of recent publications on the subject^[20-24]. The purpose of our study is to determine factors affecting the outcome of patients with cirrhosis undergoing surgical procedures under general anesthesia and to compare the capacities of the CTP and MELD scores in predicting that outcome.

MATERIALS AND METHODS

Patient selection

We conducted a retrospective review of the charts of patients with cirrhosis undergoing surgical procedures under general anesthesia between January 1999 and December 2004 at two teaching hospitals, Emory University Hospital and Emory Crawford Long Hospital.

We used the computerized medical record system to screen charts for "liver cirrhosis" and "general anesthesia" using billing codes and International Classification of Diseases-9 codes. Patients in whom the chart review did not reveal documented cirrhosis were excluded. Patients were considered to have documented cirrhosis if they had a liver biopsy confirming cirrhosis, an intraoperative finding of a cirrhotic liver during laparotomy or laparoscopy, or a combination of imaging and laboratory profiles consistent with cirrhosis. Patients undergoing surgical procedures directly involving the liver, such as liver transplantation, hepatectomy and transjugular intrahepatic portosystemic shunts were excluded. Patients for whom the preoperative CTP or MELD score could not be computed because of insufficient data were also excluded. Approval of the protocol by the Institutional Review Board of Emory University was obtained prior to the conduction of the study. The protocol conforms with the provisions of the declaration of Helsinki as revised in Edinburgh in 2000.

Data collection

Preoperative history, physical examination and laboratory values were used to calculate the CTP and MELD scores. Surgical procedures were classified by organ system and by whether the procedures are urgent or elective. Surgery was considered to be elective if the procedures could reasonably have been scheduled at a later date without the need for hospitalization in the interim. Procedures not satisfying this criterion were classified as urgent procedures. Patients were followed up for 30 post-procedure days.

The primary endpoint for the purpose of our study was the occurrence of death or hepatic decompensation during the follow-up period. Hepatic decompensation was defined as the occurrence of both clinical decompensation and biochemical evidence of worsening liver function. Occurrences of new or worsening ascites, new or worsening encephalopathy, or variceal bleeding during the follow-up period were considered to be an evidence of clinical decompensation. A rise in the international normalized ratio (INR) or bilirubin in combination with clinical decompensation was required for the endpoint to be reached.

Statistical analysis

Patient characteristics were tallied both overall and by achievement of the endpoint of death or hepatic decompensation, and descriptive statistics were calculated. Two-sample *t*-tests were used to compare means of the continuous measures between the endpoint groups. To compare categorical summaries, the chisquare test of independence was used. Where expected counts were too low, Fisher's exact test was employed. The Cochran-Armitage Test for Trend was used to examine the score system and endpoint relationships across ordinal Child classifications (A, B, C) and MELD tertiles.

These tests revealed that both score systems and procedure urgency were important factors in predicting death or decompensation. In order to more precisely quantify the roles of these variables in predicting the endpoint, a logistic regression was performed with urgency and classification score (either MELD or CTP) as independent variables, and the combined endpoint as a dependent variable. To avoid multicollinearity, the CTP and MELD scale were included in separate models.

To further characterize the relationships between score, urgency and the endpoint, we used a two-factor analysis of variance (ANOVA) to compare mean CTP and MELD scores for a model which included urgency, endpoint achievement, and their interaction.

These analyses showed that the scores significantly differed only within the urgent patient population. This directed our use of receiver-operating characteristic (ROC) plots to examine the relationship between score and patient outcome for the urgent-procedure population only. Predictive values for each scale were assessed by measuring the area under the curve (AUC). The AUCs for the CTP and MELD systems were compared using the method proposed by DeLong *et al*^[25] for two correlated ROC curves. All analyses were performed using SAS software version 9.1 (SAS Institute Inc., Cary, NC). The correlated

Table 1 Characteristics of 195 patients with cirrhosis undergoing surgical procedures under general anesthesia

	Total (n = 195)	Death or decompensation		P value	Odds ratio
		No (n = 163)	Yes (n = 32)		
Mean age (yr)	57.1 ± 11.2	57.2 ± 10.9	56.5 ± 12.5	0.73	
Gender, n (%)					
Female	79 (40.5)	65 (39.9)	14 (43.8)	0.68	
Male	116 (59.5)	98 (60.1)	18 (56.3)		
Ethnicity, n (%)					
White	159 (81.5)	135 (82.8)	24 (75.0)	0.32	
African American	27 (13.8)	20 (12.3)	7 (21.9)		
Other	9 (4.6)	8 (4.9)	1 (3.1)		
Etiology, n (%)					
Hepatitis C	73 (37.4)	61 (37.2)	12 (37.5)	0.99	
Cryptogenic	51 (26.2)	43 (26.4)	8 (25.0)	0.87	
Alcohol	35 (17.9)	26 (16.0)	9 (28.1)	0.1	
Hepatitis B	7 (3.6)	7 (4.3)	0 (0.0)	-	
Autoimmune hepatitis	5 (2.6)	5 (3.1)	0 (0.0)	-	
Primary sclerosing cholangitis	5 (2.6)	4 (2.5)	1 (3.1)	-	
Primary biliary cirrhosis	4 (2.1)	4 (2.5)	0 (0.0)	-	
A1 antitrypsin deficiency	3 (1.5)	3 (1.8)	0 (0.0)	-	
Non-alcoholic steatohepatitis	2 (1.0)	2 (1.2)	0 (0.0)	-	
Amyloidosis	2 (1.0)	2 (1.2)	0 (0.0)	-	
Hemochromatosis	1 (0.5)	1 (0.6)	0 (0.0)	-	
Cardiac cirrhosis	1 (0.5)	1 (0.6)	0 (0.0)	-	
Cystic fibrosis	1 (0.5)	0 (0.0)	1 (3.1)	-	
Unknown	5 (2.6)	4 (2.4)	1 (3.1)	-	
Mean CTP score ± SD	8.0 ± 1.9	7.8 ± 1.8	9.1 ± 1.9	0.00058	
CTP class n (%)					
A	41 (21.0)	40 (24.5)	1 (3.1)	0.0018	1
B	115 (59.0)	95 (58.3)	20 (62.5)		8.42
C	39 (20.0)	28 (17.2)	11 (34.4)		15.71
Mean MELD score ± SD	14.2 ± 6.3	13.2 ± 5.2	19.1 ± 5.2	< 0.0001	
MELD score (Tertile)					
6-11 (T1)		77 (47.2)	6 (18.8)	0.0008	1
12-15 (T2)		46 (28.2)	10 (31.3)		2.79
16-40 (T3)		40 (24.5)	16 (50.0)		5.13
Surgery sites					
Gastrointestinal	101 (51.8)	84 (51.5)	17 (53.1)	0.87	
Cardiovascular and thoracic	28 (14.4)	22 (13.5)	6 (18.8)	0.42	
Genitourinary	24 (12.3)	22 (13.5)	2 (6.3)	0.38	
Orthopedic	24 (12.3)	20 (12.3)	4 (12.5)	-	
Head and neck	18 (9.2)	15 (9.2)	3 (9.4)	-	
Surgery type					
Urgent	57 (29.2)	36 (22.1)	21 (65.6)	< 0.0001	
Elective	138 (70.8)	127 (77.9)	11 (34.4)		

CTP: Child-Turcotte-Pugh; MELD: Model for end-stage liver disease.

AUC test was implemented using the ROC SAS macro provided by DeLong *et al*^[26].

RESULTS

A total of 617 patients were identified on initial screening. Of the initial group of patients, 258 had documented cirrhosis. Forty-five patients were excluded for undergoing liver-related surgery, and 18 patients were excluded because of insufficient data. A total of 195 patients were included in the study.

Patients were mostly white with a male predominance. The mean patient age was 57.1 years. Hepatitis C was the leading etiology of cirrhosis, followed by cryptogenic cirrhosis and alcoholic cirrhosis. Most patients were CTP class B, with a mean ± SD CTP score of 8.0 ± 1.9. The mean ± SD MELD score was 14.2 ± 6.3 (Table 1). The most commonly performed procedures were

gastrointestinal. A total of 138 procedures were classified as elective and 57 as urgent. The procedures performed are detailed in Table 2.

Seventeen (8.7%) patients died during follow-up and 28 (14.4%) patients had evidence of hepatic decompensation. Among the 17 patients who died, 13 (76.5%) had evidence of hepatic decompensation as well. A total of 32 patients (16.4% of the total, 21/57 patients in the urgent surgery group and 11/138 patients in the elective surgery group) reached the study endpoint of death or decompensation in the postoperative period. The causes of death were sepsis in 47.1% (8/17), gastrointestinal bleeding in 23.6% (4/17), and sudden cardiac arrest in 5.9% (1/17) cases. The cause of death was unknown in 4 patients. New or worsening hepatic encephalopathy was observed in 20 (10.3%) patients, new or worsening ascites in 9 (4.6%) patients, and variceal bleed in 8 (4.1%) patients.

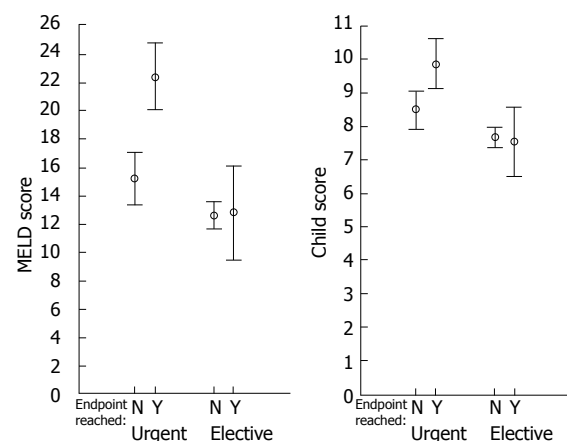
Bivariate analyses revealed a significantly different

Table 2 Surgical procedures performed in 195 patients with cirrhosis

	Total	Elective	Urgent
Procedures	195	138	57
Gastrointestinal	101	66	35
Hernia repair	34	21	13
Cholecystectomy	23	17	6
Exploratory/diagnostic	10	6	4
Biopsy of lesion	9	9	0
Colectomy	6	3	3
Abscess drainage	3	1	2
Hemorrhoid ligation	3	2	1
Pancreatectomy	3	3	0
Small bowel resection	3	0	3
Peptic ulcer oversewing	2	0	2
Appendectomy	1	0	1
Antrectomy (Billroth II)	1	1	0
Gastric bypass	1	1	0
Gastrostomy tube placement	1	1	0
Splenectomy	1	1	0
Cardiovascular and thoracic	28	25	3
Coronary artery bypass	5	5	0
Video-assisted thoracoscopic surgery	5	4	1
Abdominal aortic aneurysm repair	4	4	0
Arteriovenous fistula/graft construction	4	4	0
Cardiac valve replacement/repair	3	3	0
Lung biopsy, open	3	3	0
Pericardiectomy	2	0	2
Heart transplant	1	1	0
Pulmonary lobectomy	1	1	0
Genitourinary	24	21	3
Cystoscopy/ureteroscopy	15	12	3
Dilation and curettage	4	4	0
Hysterectomy	3	3	0
Oophorectomy	1	1	0
Transurethral resection of prostate	1	1	0
Orthopedic	24	12	12
Total hip replacement	6	6	0
Amputation	5	3	2
Infected hip prosthesis removal	4	0	4
Incision and drainage	4	0	4
Open reduction - internal fixation	3	2	1
Arthroscopy	2	1	1
Head and neck	18	14	4
Endoscopic sinus surgery	3	3	0
Laryngoscopy	3	3	0
Parathyroidectomy	3	3	0
Craniotomy	2	1	1
Facial fracture reduction	1	0	1
Mastoidectomy	1	1	0
Neck dissection	1	1	0
Parotidectomy	1	1	0
Scleral buckle	1	1	0
Teeth extraction	1	0	1
Ventriculo-peritoneal shunt	1	0	1

Table 3 Logistic regression analysis: odds ratio of death or decompensation in the postoperative period using the parameters of procedure urgency and the MELD score (A) or the CTP score (B)

	Odds ratio	95% CI	P
A: MELD model			
MELD (1 unit increase)	1.1	[1.03-1.17]	0.0044
MELD (5 unit increase)	1.58	[1.16-2.19]	
Elective (no vs yes)	4.34	[1.81-10.71]	0.0011
B: CTP model			
CTP (1 unit increase)	1.25	[1.00-1.57]	0.0554
Elective (no vs yes)	5.18	[2.20-12.22]	0.0002

**Figure 1** Mean MELD and Child scores with 95% confidence intervals, stratified by procedure urgency. A significant difference was observed in means between the patients who reached the endpoint and the patients who did not in the case of urgent surgery, but not in the case of elective surgery.

Among patients undergoing elective surgical procedures, no statistically significant difference was noted in the mean \pm SD MELD score (12.8 ± 3.9 vs 12.6 ± 4.7 , $P = 0.9$) or in the mean \pm SD CTP score (7.6 ± 1.2 vs 7.7 ± 1.7 , $P = 0.8$) between patients who reached the endpoint and patients who did not. Conversely, both the mean \pm SD MELD score and mean CTP score were higher in patients reaching the endpoint in the case of urgent procedures (MELD: 22.4 ± 8.7 vs 15.2 ± 6.4 , $P = 0.0007$; CTP: 9.9 ± 1.8 vs 8.5 ± 1.8 , $P = 0.008$, Figure 1).

When evaluating the capacities of the CTP and MELD scores to predict the occurrence of the study endpoint in the 57 patients undergoing urgent surgical procedures, the performances of both scoring systems were found to be fair: the area under the receiver operating characteristics curve (AUC) for MELD was 0.755 ± 0.066 , the AUC for CTP was 0.696 ± 0.070 (Figure 2A and B). No statistically significant difference was found between the AUC for MELD and the AUC for CTP for these patients ($P = 0.20$, Figure 2C). A MELD score of 17 had a sensitivity of 71% and a specificity of 72% in predicting the outcome of death or decompensation in urgent surgical procedures. A CTP score of 9 had a sensitivity of 81% and specificity of 47% in predicting the outcome. Among the patients undergoing urgent surgical procedures, none of the 15/57 patients with a preoperative MELD score of ≤ 11 and none of the 15/57 patients with a preoperative

trend between the proportion of patients that reached the study endpoint in each MELD tertile, as well as each CTP class ($P = 0.0008$ for MELD, $P = 0.0018$ for CTP). Those who experienced death or decompensation tended to be in the higher score classifications. Patients who reached the endpoint were also more likely to have had an urgent procedure ($P < 0.0001$). No inter-group difference was found in the other baseline characteristics (Table 1). Logistic regression analyses revealed that the MELD score, CTP score and the urgency of the procedure were significantly associated with the study endpoint (Table 3).

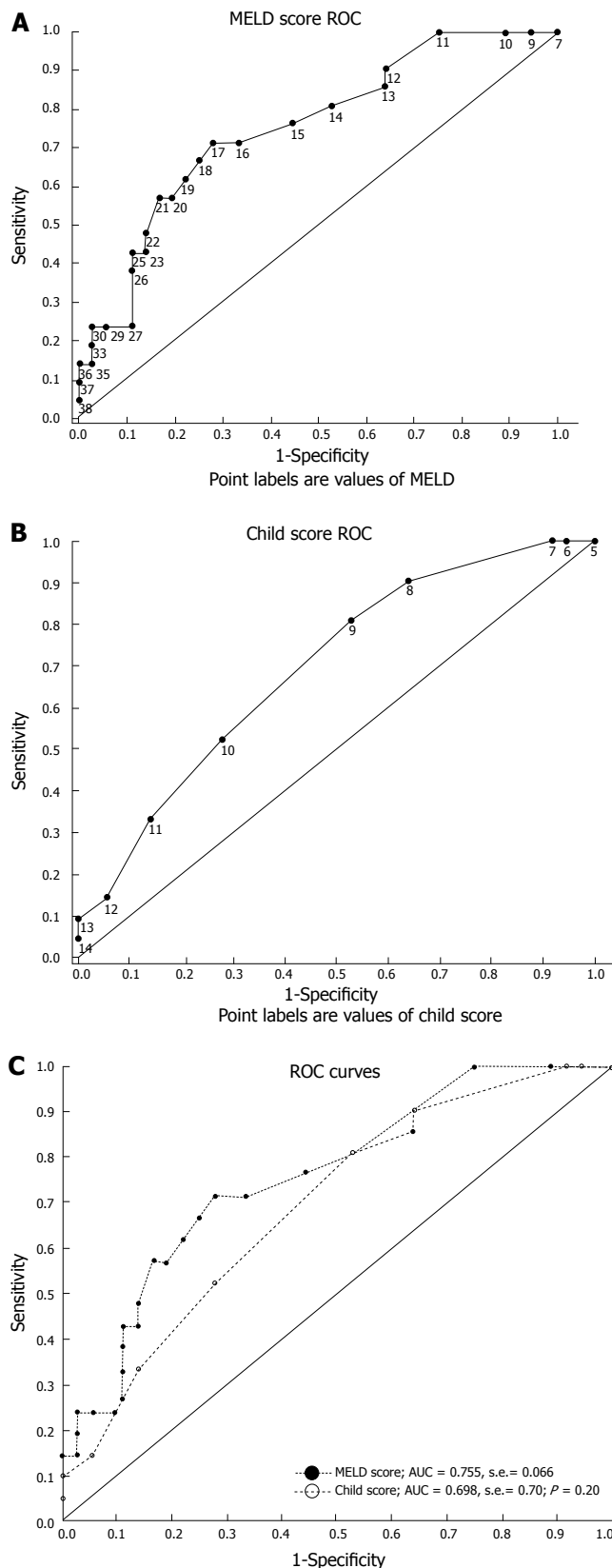


Figure 2 Receiver operating characteristics (ROC) curves for MELD score (A) and CTP score (B) as predictors of death or hepatic decompensation in the postoperative period in 57 cirrhotic patients who underwent urgent surgical procedures under general anesthesia. Panel C shows a comparison of the two curves.

CTP score of ≤ 7 had any evidence of death or hepatic decompensation in the post-operative period.

DISCUSSION

Gastroenterologists and hepatologists are often asked to evaluate patients with cirrhosis prior to undergoing non-liver-related surgical procedures for an opinion about the perioperative risk entailed and potential ways to reduce that risk. Unfortunately, the available evidence to support answers to these questions is relatively limited and is uniformly based on retrospective data.

Early studies addressing preoperative evaluation in cirrhosis identified the Child-Pugh score as a useful preoperative parameter in predicting surgical risk in patients undergoing abdominal surgery with a mortality rate of 10% for Child class A patients, 30%-31% for Child class B, and 76%-82% for Child class C patients^[27,28]. Compared to the Child score, the MELD score has an advantage of relying entirely on objective parameters for its computation, since it does not require an evaluation of the degree of ascites and the degree of encephalopathy. Also, the MELD score is a variable with a wider range of possible numeric values (6 to 40 for MELD compared to 5 to 15 for Child), thereby potentially allowing a better discrimination between patients with different degrees of hepatic dysfunction. Perkins *et al*^[22] did not find a difference in the performances of the MELD score and the Child score in predicting the morbidity of cholecystectomy in 33 patients with cirrhosis. The authors favored the use of the MELD score because it is more objectively defined, and suggested a MELD score of 8 as a cut-off point that predicts an increased morbidity. Data about the urgency of the procedure was not provided^[22]. In evaluating the outcome of 66 patients with cirrhosis undergoing predominantly elective cardiac surgery, Suman *et al*^[21] found that the Child score and the MELD score were comparable in predicting the occurrence of hepatic decompensation in the postoperative period. The Child score cut-off point of 7, however, had a higher sensitivity in predicting mortality than a MELD cut-off of 13. In reviewing their experience with cardiac surgery in 27 patients with cirrhosis, Filsoufi *et al*^[29] noted that the Child score was a better predictor of hospital mortality than the MELD score. On the other hand, Befeler *et al*^[23] found the MELD score to be a better predictor of poor outcome than the Child class in 53 cirrhotic patients undergoing abdominal surgery, although in this case the MELD score was compared to Child class categories, and not to the numeric value of the Child score.

Our study included 195 patients with documented cirrhosis undergoing various surgical procedures under general anesthesia. The patients included in our study had fairly advanced liver disease with 57.4% of patients having a MELD score ≥ 12 and 79% being Child class B or C. The operative mortality (8.7%) that we observed is similar to the mortality observed in a large series of patients with cirrhosis undergoing surgery^[16].

We found the urgency of the procedure to be a powerful predictor of the outcome, with the occurrence of the combined endpoint of death or hepatic decompensation being observed more than 4 times more often in the patients undergoing urgent surgery compared to the patients undergoing elective surgery. This effect

persisted when controlling for the MELD or Child score in multivariate analysis (Table 3). Furthermore, when the data was stratified by the urgency of the procedure, neither the MELD nor the Child score was able to predict the outcome of elective surgery. This may be due to the lower event rate in patients undergoing elective surgery, as might be expected. It is conceivable as well that if a larger number of patients were included, the MELD score and Child score might have had a better discriminatory ability in predicting the outcome of elective surgery. This finding emphasizes, however, the importance of making a distinction between elective procedures and urgent procedures when studying the effect of variables on surgical outcome in patients with cirrhosis. In prior similar studies addressing predictors of operative outcomes in cirrhotic patients, this important distinction was not always made.

In the group of patients undergoing urgent surgery, the MELD score and Child score performed equally in predicting the occurrence of death or hepatic decompensation in the postoperative period. The performance of both scoring systems was only fair in that regard, with an AUC of 0.755 for MELD and 0.696 for Child, which is reflected in the lack of a cut-off point with both a high sensitivity and a high specificity in predicting the outcome for either scoring system. This is unlike the case of other studies where higher AUCs were noted^[21,22]. Studies with higher AUC values had studied specific surgical procedures, and the reason for the lower AUC in our study is likely to be the fact we studied a variety of procedures with different inherent risks to the patient. This problem could be circumvented by devising a classification system of surgical procedures by their inherent risk to patients with liver dysfunction, similar to the established system used in the case of perioperative risk assessment in cardiovascular disease^[30]. In their seminal study on perioperative risk in patients with cirrhosis, Ziser *et al*^[16] provided some useful guidance in this direction; however, this remains an area in need of further study.

We noted that among patients in our study undergoing urgent surgery with either a MELD score ≤ 11 or a Child ≤ 7 , none had evidence of death or decompensation postoperatively. Patients falling into this low-risk category represented a substantial proportion (20/57, 35.1%) of the total number of patients undergoing urgent surgery, suggesting that these cut-off points may be useful in practice.

It is likely that the role of preoperative evaluation in patients with cirrhosis is more critical in the case of elective surgical procedures than it is in the case of urgent procedures. In many instances, patients undergoing urgent surgery may have no reasonable alternative to surgery, and may have little time for any preoperative intervention that could potentially reduce the risk of complications. Therefore, the best opportunity for Gastroenterologists or Hepatologists to intervene in a manner that may reduce surgical risk or to provide useful guidance as to whether or not to proceed with surgery is probably in the case of elective surgery rather than urgent surgery. We could not demonstrate that MELD score and Child score were helpful in determining the outcome of elective surgery.

Furthermore, larger studies are needed to better define the roles of the Child and MELD scores in predicting the outcome of surgical procedures in patients with cirrhosis, particularly in the case of elective surgery.

COMMENTS

Background

Patients with cirrhosis who undergo surgeries under general anesthesia are at an increased risk of complications. The Child-Turcotte-Pugh (CTP) class has been used to assess the risk of surgery in patients with cirrhosis. The model for end-stage liver disease (MELD) score is a more recently devised tool to assess the severity of liver disease.

Research frontiers

Previous studies comparing the CTP score and the MELD score in predicting the outcome of surgery in cirrhosis yielded variable results, generally pointing in the direction of both scoring systems being good predictors of the outcome of non-hepatic surgery in cirrhosis. Only few studies made the distinction between elective and urgent surgery.

Innovations and breakthroughs

The urgency of the surgical procedure was found to be a major predictor of the outcome, with the complication rate of elective procedures being generally low. In elective procedures, the CTP score and MELD score were not predictive of the outcome. In urgent procedures, the MELD score and CTP score performed equally in predicting an adverse outcome.

Applications

The MELD score and CTP score could be used alternatively in evaluating the risk of an urgent surgical procedure. Studies are needed to assess the risk of elective procedures in patients with cirrhosis.

Terminology

The CTP and MELD scores are both measures of the severity of liver disease in cirrhosis. The MELD score is computed entirely using objective laboratory data. The computation of the CTP scores requires an assessment of non-objective parameters, including the severity of ascites and of encephalopathy.

Peer review

In this retrospective study, the authors determined the factors affecting the outcome of surgeries under general anesthesia in patients with cirrhosis and compared the capacities of the CTP and MELD scores to predict that outcome. They concluded that CTP and MELD scores performed equally, but only fairly in predicting the outcome of urgent surgical procedures.

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Short-term overlap lamivudine treatment with adefovir dipivoxil in patients with lamivudine-resistant chronic hepatitis B

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Abstract

AIM: To evaluate the efficacy of short-term overlap lamivudine therapy with adefovir in patients with lamivudine-resistant and naïve chronic hepatitis B, we compared patients receiving overlap therapy with those receiving adefovir alone.

METHODS: Eighty patients who had received lamivudine treatment for various periods and had a lamivudine-resistant liver function abnormality were enrolled. Forty of these patients received adefovir treatment combined with lamivudine treatment for ≥ 2 mo, while the other 40 received adefovir alone. We assessed the levels of hepatitis B virus (HBV) DNA at 0, 12 and 48 wk and serum alanine aminotransferase (ALT) levels after 0, 12, 24 and 48 wk of adefovir treatment in each group.

RESULTS: We found serum ALT became normalized in 72 (87.5%) of the 80 patients, and HBV DNA decreased by $\geq 2 \log_{10}$ copies/mL in 60 (75%) of the 80 patients at the end of a 48-wk treatment. HBV DNA levels were not significantly different between the groups. The improvements in serum ALT were also not significantly different between the two groups.

CONCLUSION: These findings suggest short-term overlap lamivudine treatment results in no better virological and biological outcomes than non-overlap adefovir monotherapy.

INTRODUCTION

Hepatitis B virus (HBV) infection is the main cause of various liver diseases, such as acute and chronic hepatitis, cirrhosis and hepatocellular carcinoma^[1,2]. To prevent progression into end stage liver diseases, effective and reliable treatment of chronic hepatitis B has been urgently needed. Five regimens have been used for the treatment of chronic hepatitis B. The approved drugs are interferon (conventional and pegylated), lamivudine, adefovir dipivoxil and entecavir. Lamivudine has been effective and tolerable in chronic hepatitis B, but grave drug-resistant mutations developed up to 65% after 5 years of treatment^[3]. Unlike conventional interferon, pegylated interferon treatment could lead to similar or more efficient virological responses than lamivudine, but unwillingness to injection and a number of side effects have prevented its further active usage. However, recent reports showed more optimizing results than ever^[4].

Adefovir dipivoxil is a nucleotide analogue which has been shown to have potent anti-viral activity and side effects similar to those of lamivudine. It also has anti-viral effects against both naïve and lamivudine-resistant cases^[5,6]. Because of provisions in Korean national medical insurance which restrict the use of adefovir to 'rescue' treatment in cases of lamivudine resistance, adefovir dipivoxil is generally prescribed to patients with lamivudine-resistant HBV in Korea^[7,8].

Peter *et al*^[9] suggested the biological and virologic responses were comparable between combinations and adefovir monotherapy in patients with lamivudine-resistant HBV. However, aggravation of hepatitis is more common in patients receiving adefovir monotherapy. Therefore, an overlap period of 2-3 mo was recommended due to the risk of aggravation of liver function during the transition time.

We analyzed the differences between short-term overlap therapy with lamivudine and adefovir and adefovir monotherapy based on viral and blood chemical studies to elucidate the effectiveness of overlap.

MATERIALS AND METHODS

Clinical characteristics of the patients

From March 2004 to May 2006, 80 chronic hepatitis B patients were sequentially enrolled from admission and/or outpatient department and/or the Daejeon St. Mary's Hospital in the Medical College of the Catholic University of Korea. All patients gave written informed consent to participate in this study. All patients had been prescribed lamivudine for chronic hepatitis B and had developed lamivudine-resistant YMDD mutant HBV. YMDD mutation was confirmed by polymerase chain reaction (PCR) in our clinical pathology department. Prior to enrollment, all patients had a serum HBV DNA concentration above 10^5 copies/mL and elevated serum ALT above 80 IU/L. The exclusion criteria were as follows: Patients showing serologic and/or radiological evidence of alcoholic hepatitis or cirrhosis; patients who did not comply with a regular check up of serological and/or virological markers; patients with drug histories, such as herb medications.

Patients received 10 mg adefovir dipivoxil (HepseraTM) orally once a day after enrollment, for 48 wk. Forty patients also received 100 mg lamivudine (ZeffixTM) for 2 mo.

Virological and chemical assays

Serum ALT levels were assessed at intervals of 3 mo and HBV DNA levels were also assessed at 0, 24 and 48 wk. HBeAg and anti-HBe (HBeKit, Beijing North Institute of Biological Technology, Beijing, China) (Quantum Cobra Series II, Packard Inst. Co. Meriden, CT, USA) were assayed at 0, 12 and 48 wk. Serum HBV DNA was examined using an HBV DNA PCR instrument (Covas AmplicorTM, Roche Diagnostics systems, Mannheim, Germany).

Statistical analysis

Data were recorded as mean \pm SD and analyzed by paired *t*-tests. A *P* value of < 0.05 was considered to be statistically significant.

RESULTS

Baseline characteristics of patients

The baseline characteristics of the enrolled patients are shown in Table 1. There were no significant differences in clinical, virological and chemical characteristics between the groups. All enrolled patients completed the 48-wk adefovir treatment schedule.

Table 1 Baseline characteristics of patients in each group

	Overlap	Non-overlap
Age (yr)	43.3 \pm 8.9	43.7 \pm 13.0
Male/female ratio	32/8	29/11
Baseline ALT (IU/L)	188.4 \pm 74.1	209.6 \pm 130.6
Baseline HBV DNA (log ₁₀ copy/mL)	6.9 \pm 0.6	6.9 \pm 0.7
Median lamivudine treatment (mo)	21.5	20.3
HBeAg positive/negative	22/18	25/15

Table 2 Comparison of mean serum ALT levels in each group

	wk after adefovir treatment (IU/L)				
	0	12	24	36	48
Overlap	188.4 \pm 74.1	77.3 \pm 53.9	78.3 \pm 90.2	34.2 \pm 13.4	25.9 \pm 9.9
Non-overlap	209.6 \pm 130.6	78.9 \pm 40.8	43.6 \pm 24.9	34.2 \pm 8.7	24.0 \pm 8.7
<i>P</i> -value	NS	NS	NS	NS	NS

NS: Not statistically significant.

Table 3 Comparison of median serum HBV DNA levels in each group

	wk after adefovir treatment (Log ₁₀ copies/mL)		
	0	12	48
Overlap	6.9 \pm 0.6	5.5 \pm 1.4	3.8 \pm 1.0
Non-overlap	6.9 \pm 0.7	4.2 \pm 1.2	3.4 \pm 1.4
<i>P</i> -value	NS	NS	NS

NS: Not statistically significant.

Comparison of ALT and HBV DNA levels between overlap and non-overlap

At the end of the study schedule, serum ALT level was normalized in 72 (87.5%) of the 80 patients. Overall, the HBV DNA level was decreased by more than 2 log₁₀ copies/mL in 60 (75%) of the 80 patients. HBeAg became negative in 6 (12.7%) of the 47 baseline HBeAg-positive patients. HBeAb seroconversion developed in none of the baseline HBeAg-positive patients.

Serum ALT levels at intervals of 3 mo showed no statistically significant difference between the groups, and ALT level flare up after adefovir monotherapy was seen in only 4 (10%) of the non-overlap patients (*vs* 7.5% of the overlap patients) (Table 2). HBV DNA levels at noted schedules also showed no specific statistically significant difference between the groups (Table 3).

DISCUSSION

There are many suggestions and debates about the management of patients with lamivudine-resistant mutant HBV. However, no obvious consensus has emerged so far. Due to the reported high resistance to lamivudine treatment, many patients who received lamivudine as a therapy for chronic hepatitis B in Korea have had their prescription changed to other anti-viral agents, especially adefovir^[7]. Adefovir has a similar potency and side effects,

but is associated with lower resistance than lamivudine^[5,6]. Whether lamivudine should be prescribed for short-term treatments in combination with adefovir has been a matter of controversy^[9,10]. Thus, we performed a prospective study to elucidate the efficacy of a short-term overlap lamivudine treatment. Although a short-term overlap lamivudine therapy was recommended due to the acute flare ups in serum ALT level reported by Peter *et al*^[9], similar ALT levels were seen in the two groups in our study.

All patients enrolled in our study had compensated chronic liver diseases and no patients had decompensated liver functions after a 48-wk follow up. HBV DNA levels and serum ALT levels were decreased sequentially by adefovir treatment with or without short-term overlap. Therefore, adefovir dipivoxil treatment was effective against lamivudine-resistant chronic hepatitis B.

Hadziyannis *et al*^[11] recently reported adefovir monotherapy for HBeAg-negative chronic hepatitis B for up to 5 years was well tolerated, increasing improvement in the fibrosis of the liver, durable suppression of HBV DNA, and up to 29% resistance. Because of good results following long-term therapy with adefovir with or without short-term overlap lamivudine treatment, adefovir monotherapy without overlap lamivudine therapy might be validated.

Recently, Liu *et al*^[12] reported that an overlap of lamivudine and adefovir treatments for more than 2 mo leads to better virological but not biochemical outcomes in patients with lamivudine-resistant HBV than adefovir treatment alone. However, their study population was smaller than ours and comprised non-randomized cases. Our data showed short-term overlap lamivudine therapy was not superior to non-overlap adefovir monotherapy group with regard to virological and/or biochemical laboratory data. HBeAg loss and HBeAg seroconversion rates were similar to those reported in previous studies by Locarinni *et al*^[13]. Both HBeAg loss and HBeAg seroconversion were no better than lamivudine.

Akyildiz *et al*^[14] also recently reported there was no significant difference between patients with lamivudine resistant chronic hepatitis B treated with adefovir alone and those treated with adefovir in combination with lamivudine for three mo. Therefore, the authors asserted it was not necessary to continue lamivudine treatment while switching to adefovir dipivoxil monotherapy. These results are consistent with ours, although it is not perfectly obvious to assert that switching to adefovir dipivoxil monotherapy with an instant cessation of lamivudine. Our study also has several limitations, such as an insufficient study population and/or non-randomized with blinded state. Moreover, follow up longer than 48 wk is ongoing state.

In summary, our results suggest there is no greater benefit of short-term overlap lamivudine treatment than non-overlap adefovir monotherapy. An overlap of more than 2 mo showed no better virological and/or biochemical results. Therefore, starting adefovir monotherapy immediately instead of applying a short-term overlap might be an efficient choice in cases of lamivudine-resistant HBV. Furthermore, new anti-viral agents with higher potency, durability and/or HBV seroconversion rate should be devised in the near future.

COMMENTS

Background

To prevent progression into end-stage liver diseases, effective and reliable treatment for chronic hepatitis B has been urgently needed. Adefovir dipivoxil is effective in patients with lamivudine-resistant and naïve chronic hepatitis B. The efficacy of short-term overlap treatment with lamivudine and adefovir is not well known. We compared patients who received short-term overlap therapy with those who received adefovir only to evaluate the efficacy of short-term overlap lamivudine therapy.

Research frontiers

Peter *et al* suggested the biological and virologic responses were comparable between combinations of adefovir and lamivudine therapies and adefovir monotherapy in patients with lamivudine-resistant Hepatitis B virus (HBV). However, aggravation of hepatitis was more common in patients receiving adefovir monotherapy. Therefore, an overlap period of 2-3 mo was recommended due to the risk of aggravation of liver function during the transition time. Liu *et al* reported an overlap of lamivudine and adefovir for longer than 2 mo leads to better virological but not biochemical outcomes in patients receiving adefovir for lamivudine-resistant HBV. Akyildiz *et al* reported there was no significant difference between patients with lamivudine-resistant chronic hepatitis B receiving adefovir alone and those receiving adefovir in combination with lamivudine for three months. Therefore, the authors asserted it was not necessary to continue lamivudine treatment while switching to adefovir dipivoxil monotherapy.

Innovations and breakthroughs

Our study has a larger study population and a more objective differentiation of liver function than other studies. However, our study also has several limitations, such as an insufficient study population and/or non-randomized with blinded state.

Applications

There are needs for longer and larger scales of study in this field. Although the recent article from Hadziyannis *et al* showed the superiority of combination therapy, more studies are needed to study the comparison between switching with or without overlap and combination therapy of adefovir and lamivudine.

Terminology

Overlap of lamivudine and adefovir: Use of two regimens at the same time for several months to avoid viral flare up; Switching: When mutant HBV strains resistant to lamivudine develop, other regimens (such as Adefovir, Entecavir and Clevudine) can be substituted for lamivudine.

Peer review

This study evaluates the efficacy of short-term overlap of lamivudine and adefovir in patients with lamivudine-resistant chronic hepatitis B. Compared with non-overlap adefovir monotherapy, short-term overlap lamivudine treatment show no better virological and biological outcomes. This can be interesting for the gastroenterologists and may represent a potentially efficacious approach to the clinical management of acute HBV infection.

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Cyclooxygenase 2 polymorphism and colorectal cancer: -765G>C variant modifies risk associated with smoking and body mass index

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is not associated with an increased risk of CRC, -765GG genotype appears to be related to an increased risk in the presence of smoking and higher BMI.

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Key words: Colorectal cancer; Cyclooxygenase 2; Polymorphism; Smoking; Body mass index

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Xing LL, Wang ZN, Jiang L, Zhang Y, Xu YY, Li J, Luo Y, Zhang X. Cyclooxygenase 2 polymorphism and colorectal cancer: -765G>C variant modifies risk associated with smoking and body mass index. *World J Gastroenterol* 2008; 14(11): 1785-1789 Available from: URL: <http://www.wjgnet.com/1007-9327/14/1785.asp> DOI: <http://dx.doi.org/10.3748/wjg.14.1785>

Abstract

AIM: To explore whether cyclooxygenase 2 (*COX-2*) -765G>C polymorphism is associated with susceptibility of colorectal cancer (CRC) and to evaluate the risk of colorectal cancer in relation to environmental exposures and polymorphism.

METHODS: We conducted a case-control study of 137 patients with colorectal cancer and 199 cancer-free controls in northeast China. Multivariate logistic regression analysis was performed to calculate the adjusted odds ratio (OR) and 95% confidence interval (95% CI).

RESULTS: The -765G>C polymorphism was not independently associated with CRC risk. However, risk associated with the polymorphism differed by smoking and body mass index (BMI). Smoking and BMI associated risks were stronger among those with -765GG genotype, showing that smokers had a 2.682-fold greater risk of CRC than nonsmokers (51/43 vs 68/126, $P = 0.006$). Compared to those with a normal body mass index (BMI 18.5-22.9), those with overweight (BMI 23-24.9) had a 3.909-fold higher risk of CRC (OR = 3.909, 95% CI = 2.081-7.344; $P < 0.001$), while those with obesity (BMI > 25) had a 2.031-fold higher risk of CRC (OR = 1.107, 95% CI = 1.107-3.726; $P = 0.022$).

CONCLUSION: Although *COX-2* -765G>C polymorphism

INTRODUCTION

Cyclooxygenases (COXs) are rate-limiting enzymes for prostaglandin production^[1]. Two isoforms were described, the constitutively expressed *COX-1* and the inducible isoform *COX-2*^[2]. *COX-2* is expressed at low levels in most tissues but high in inflammatory states^[3], and is induced by a variety of stimulators including cytokines^[4], growth factors^[5], as well as tumor promoters^[6]. Prostaglandin synthesis by the *COX-2* is regulatory compounds that play major roles in the inflammatory response^[2]. Chronic inflammation is responsible for the development and progression of many common cancers. It is well established that patients with inflammatory bowel disease are at increased risk of developing colorectal cancer (CRC)^[7,8].

COX-2 promoter region contains multiple regulatory elements, such as nuclear factor-Kb (NF-κB) binding site, nuclear factor interleukin-6 (NF-IL6)/CCAAT/enhancer-binding protein (C/EBP) binding site, cyclic AMP-response element (CRE) and activation protein 1 (AP-1). The regulation of *COX-2* gene expression could involve complex interaction among them^[9]. Growing evidences indicate that genetic variants in the promoters of *COX-2* gene may modulate risk for breast cancer^[10],

gastric adenocarcinoma^[11], prostate cancer^[12] and colorectal adenoma^[13]. A common promoter variant, -765G>C (rs20417), a G to C transversion resulting in significantly lower promoter activity, and reduced levels of C-reactive protein (CRP), a systemic marker of inflammation^[14].

In this study, we explored the association between the COX-2 promoter variant (-765G>C) and the risk for CRC. In view of that the environmental exposures are associated with an increased risk of CRC, we investigated interactions between the polymorphism and the environmental factors such as smoking status, intake of alcohol and BMI.

MATERIALS AND METHODS

Study population

Unrelated subjects from Shenyang of China were enrolled for case-control studies of risk factors for CRC. The trial recruited 137 CRC patients and 199 healthy control subjects. Cases were patients with a histologically confirmed diagnosis of CRC in the First Affiliated Hospital of China Medical University and Shenyang Hospital of Anal Diseases, between 2005 and 2006. The CRC patients included 71 men and 66 women, and their median age was 61.29 years. The patients were grouped according to the TNM-classification (UICC) based on the postoperative histopathology evaluation.

Control subjects were randomly selected among the people admitted to the same hospital during the same period. There were 104 men and 95 women; the median age was 60.65 years. They had no histories of cancer.

All subjects were consent to participate in the study, and allow their blood samples to be analyzed. Detailed information on risk factors including tobacco and alcohol consumption and higher BMI were obtained with a baseline questionnaire. This study used the suggested WHO BMI cutoff points for Asians to assess several variables, respondents whose BMI was less than 23 kg/m² were categorized as having normal weight, and respondents whose BMI was 23 kg/m² or higher were categorized as being overweight and obese).

Genotyping

DNA extraction: Five mL of venous blood was collected from each subject. Genomic DNA was extracted using proteinase K digestion followed by a salting out procedure.

SNP genotyping: The COX-2 -765G>C genotypes were determined by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) assay. The primers were 5'-GGCTGTATATCTGCTCTATATGC-3' (forward) and 5'-CCGCTTCCTTTGTCCATCAG-3' (reverse). The target sequence was amplified in a 20 µL volume containing 20 ng DNA template, 2.0 µL 10 × PCR buffer, 0.5 U Taq-DNA-polymerase, 20 pmol of each primer and 1.6 µL 2.5 mmol/L dNTP. Amplification was performed for 1 min at 94°C and followed by 35 cycles of 30 s at 94°C, 30 s at 59°C, and 30 s at 72°C, and with a final step at 72°C for 1 min. The PCR products were then digested with *AciI* (New England BioLabs) and separated on 8% polyacrylamide gel electrophoresis. After electrophoresis, homozygous C allele was represented by a DNA band sized at 306 bp, whereas homozygous G allele

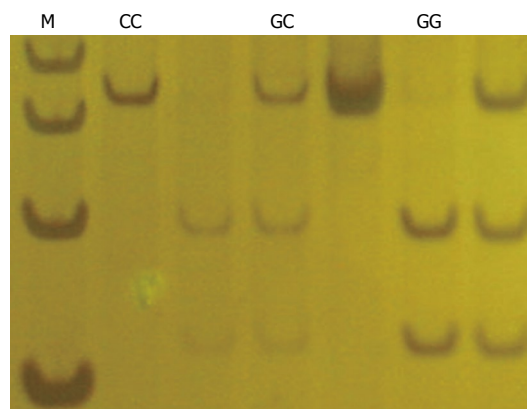


Figure 1 Genotyping of COX-2 -765G>C polymorphism by PCR-RFLP.

was represented by a DNA band sized at 118 bp and 188 bp, and heterozygotes sized at 306 bp, 118 bp and 188 bp (Figure 1).

Statistical analysis

Odds ratios (ORs) and 95% confidence intervals (CIs) were estimated by logistic regression analyses based on the comparison of genotypes between CRC patients and healthy controls using SPSS version 13.0, adjusting for the potential confounders such as age, gender, tobacco use, alcohol use, and BMI. The asymptomatic Pearson's χ^2 test was used to assess Hardy-Weinberg equilibrium. Data were considered significant when $P < 0.05$.

RESULTS

Characteristics of the study population and the association with CRC are presented in Table 1. There were no significant differences in terms of distributions in age and gender between the cases and controls ($P = 0.325$ and 0.951 , respectively), but the cases tended to have a higher body mass index ($P < 0.001$) and more likely to smoke cigarettes ($P < 0.001$).

Distribution of the COX-2 -765G>C polymorphism genotypes in CRC patients and control subjects is shown in Table 2. Distribution of genotypes in controls was in good agreement with Hardy-Weinberg equilibrium ($P = 0.838$), so did in cases ($P = 0.651$). Overall, there was no association between CRC risk and COX-2 -765G>C genotype. Similarly, no significant association was observed between -765G>C polymorphism and clinicopathological parameters among CRC patients (Table 3).

Risk associated with the COX-2 -765G>C variant differed by smoking and BMI (Table 4). There was a positive association between cigarette smoking and development of CRC in the study population. The -765GG genotype in smokers was associated with a relatively increased risk of colorectal cancer compared to non-smokers (OR = 2.682, 95% CI = 1.336-5.385; $P = 0.006$). The intake of alcohol was not positive for CRC development in this study. The effect of -765GG genotypes on CRC development was observed when individuals were stratified by BMI status. Compared with a normal BMI (18.5-22.9), those overweight (BMI 23-24.9)

Table 1 Characteristics of cases and controls

	Controls/cases	OR 95% CI
Sex		
Male	101/70	1
Female	98/67	0.986 (0.638-1.524)
Age (yr)		
≤ 60	98/60	1
> 60	101/77	1.245 (0.804-1.928)
Smoking status		
Non-smoker	147/75	1
Smoker	52/62	2.337 (1.473-3.708) ^b
Alcohol duration (yr)		
Never	168/103	1
1-15	16/14	1.427 (0.669-3.046)
> 15	15/20	2.175 (1.066-4.437)
Body mass index (kg/m ²)		
18.5-22.9	101/35	1
23-24.9	38/54	4.101 (2.329-7.220)
> 25	60/48	2.309 (1.345-3.962) ^d

^b*P* < 0.001 *vs* non-smoker group (two-sided χ^2 test); ^d*P* < 0.001 *vs* normal BMI group (two-sided χ^2 test).

Table 2 *COX-2* -765G>C genotypes and risk of CRC

	Controls/cases	OR ¹ 95% CI
<i>COX-2</i> genotype		
GG	169/119	1
GC	29/17	0.867 (0.461-1.632)
CC	1/1	

¹OR for GC/CC genotypes *versus* GG genotype and adjusted for age, gender, smoking status, alcohol consumption and body mass index.

had a 3.909-fold higher risk of CRC (OR = 3.909, 95% CI = 2.081-7.344; *P* < 0.001), while the obese (BMI > 25) had a 2.031-fold higher risk of CRC (OR = 1.107, 95% CI = 1.107-3.726; *P* = 0.022).

DISCUSSION

Numerous studies suggest that *COX-2* plays an important role in the development of CRC^[15]. However, the results of this study indicated that *COX-2* -765G>C polymorphism was not associated with CRC in this study population, but smoking and BMI may modify the risk of CRC in *COX-2* -765GG genotype.

Although the over-expression of *COX-2* is closely related to the metastasis and invasion of CRC^[16], and the *COX-2* -765G>C polymorphism located in the putative Sp1 binding site may reduce promoter activity responsible for increasing susceptibility to CRC, our study failed to detect an association between this polymorphism and CRC.

Our null findings are consistent with the previous report of an absence of an association between -765G>C polymorphism and CRC in Spaniards^[17]. Similarly, a study in Singapore Chinese suggests that the -765G>C genotype distribution in CRC patients and healthy controls was comparable, but a significant association between genotype and risk was observed among consumers of higher n-6 PUFAs^[18].

Table 3 Relationship between C-1562T and R279Q genotypes and clinicopathological features of CRC

	-765G>C GG/GC+CC	OR ¹ 95% CI
Age (yr)		
< 60	49/11	1
≥ 60	70/7	0.445 (0.161-1.230)
Sex		
Male	60/10	1
Female	59/8	0.814 (0.300-2.204)
Lymph node metastasis		
N(-) (<i>n</i> = 87)	75/12	1
N(+)	41/5	0.762 (0.251-2.314)
TNM classification		
Stage I	22/4	1
> Stage II	94/13	0.761 (0.226-2.558)
External membrane invasion		
(+)	91/11	1
(-)	25/6	1.985 (0.668-5.898)

¹The data were analyzed by Fisher's exact test.

Table 4 *COX-2* genotypes and risk for CRC in association with smoking status, alcohol duration and BMI

		-765GG		-765GC+CC
	Controls/cases	OR (95% CI)	Controls/cases	OR (95% CI)
Smoking status				
Never	126/68	1	21/7	1
Smoker	43/51	2.682 (1.336-5.385) ^b	9/11	7.963 (0.615-103.051)
Alcohol duration (yr)				
Never	143/89	1	25/14	1
1-15	13/12	0.750 (0.303-1.855)	3/2	0.713 (0.044-11.599)
> 15	13/18	0.810 (0.259-2.531)	2/2	0.199 (0.005-7.669)
Body mass index (BMI)				
18.5-22.9	86/31	1	15/4	1
23-24.9	32/46	3.909 (2.081-7.344)	6/8	5.128 (0.867-30.320)
> 25	51/42	2.031 (1.107-3.726) ^d	9/6	6.281 (0.864-45.631)

^b*P* = 0.006 *vs* non-smoker group, ^d*P* < 0.001 *vs* normal BMI group (All estimates are multivariate adjusted for age, gender, smoking status, alcohol consumption and body mass index).

In contrast, Tan *et al.*^[19] obtained contradictory positive results that the increased risk for CRC was associated with the *COX-2* -765GC genotype in Chinese population. To explain this discrepancy, several points should be considered. Firstly, the different result in association between *COX-2* -765G>C polymorphism and CRC may be due to the different study population. All our subjects were unrelated Han Chinese subjects, and drawn from a population pool in the northern part of China. Secondly, as the *COX-2* -765C allele has lower promoter activity than the G allele, the over-expression of the *COX-2* may lead to a higher risk of CRC and the G allele may be protective for CRC. In addition, the small sample size in the present study could not detect minor effect of the polymorphism on the development of CRC.

Substantial evidences indicate that significant exposure to cigarette smoke is associated with an

elevated risk for CRC and could be a factor of an early onset of CRC^[20,21]. Cigarette smoke extract could promote tumor growth directly on colon cancer cells, the effect could likely be mediated by activation of COX-2 and up-regulation of the expression of VEGF, resulting in the induction of cellular proliferation and angiogenesis^[22]. In our analysis, the COX-2 -765GG genotype in smokers was associated with a significant increase in the risk of CRC compared to non-smokers.

Obesity is also a risk factor for CRC^[23,24], and it has been shown that body mass index was independent significant predictors of CRC^[25]. In this study, a significant association was observed for interactions between the polymorphism and BMI and CRC risk, a BMI of $> 23 \text{ kg/m}^2$ was associated with an increase in the risk of CRC among -765GG genotype carriers. These findings support the hypothesis that smoking and BMI are significant risk factors for CRC. A stronger gene-environment interaction in CRC is expected.

The main limitation of our study is the lack of information on use of COX-2 inhibitors, which may cause bias in the effect of COX-2 on CRC. Another weakness of our study is the relative small study size, which may result in less precise estimation of gene-environment interaction.

In summary, our study found no association between COX-2 -765G>C polymorphism and CRC. However, our findings suggest that individuals with the -765GG genotype may be more sensitive to cigarette smoking and a higher BMI ($> 23 \text{ kg/m}^2$), perhaps attributable to the increased enzymatic activity of COX-2, and increased risk of CRC. This result is consistent with the observation that environmental factors were associated with development of CRC and that the association with smoking and BMI may differ by COX-2 -765G>C polymorphism.

COMMENTS

Background

Genetic and environmental factors are important in determining the susceptibility to colorectal cancer (CRC). COX-2, which is an enzyme responsible for the formation of prostaglandin H₂ from arachidonic acid, regulates angiogenesis and plays a critical role in tumor progression and aggressiveness. To investigate the role of COX-2 in CRC development, the authors conducted a case-control study of CRC in northeast China.

Research frontiers

Single nucleotide polymorphisms of COX-2 gene may affect the risk of cancer formation in humans, and cancer is a complex process involving genetic as well as environmental factors. The authors conducted this study to investigate the association between COX-2 polymorphism and CRC and to evaluate the potential interaction with exposures to smoking and BMI.

Innovations and breakthroughs

There are several reports on the association between COX-2 polymorphisms and CRC. In this study, although there was no association between CRC risk and COX-2 -765G>C genotype, risk associated with the COX-2 -765G>C variant differed by smoking and BMI. A stronger gene-environment interaction in CRC was observed.

Applications

In the present study, COX-2-765GG genotype appears to be associated with an increased risk in the presence of smoking and higher body mass index (BMI). The results can be used to the etiological studies and prevention of colorectal cancer.

Terminology

Single nucleotide polymorphisms or SNPs (pronounced "snips") are DNA sequence variations that occur when a single nucleotide (A, T, C or G) in the genome sequence is altered. Restriction fragment length polymorphism (RFLP) is a technique by which organisms may be differentiated by analysis of patterns derived from cleavage of their DNA.

Peer review

The relationship between a polymorphism in cyclooxygenase-2 (COX-2) promoter region and the risk of colorectal cancer was studied in this manuscript. Although the studied polymorphism failed to show an association with colorectal cancer risk, the authors argued for positive association between this polymorphism and the risk of colorectal cancer in addition to tobacco use, alcohol intake, and obesity.

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RAPID COMMUNICATION

Herbal compound 861 regulates mRNA expression of collagen synthesis- and degradation-related genes in human hepatic stellate cells

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Abstract

AIM: To identify the role of herbal compound 861 (Cpd 861) in the regulation of mRNA expression of collagen synthesis- and degradation-related genes in human hepatic stellate cells (HSCs).

METHODS: mRNA levels of collagen types I and III, matrix metalloproteinase 1 (MMP-1), matrix metalloproteinase 2 (MMP-2), membrane type-1 matrix metalloproteinase (MT1-MMP), tissue inhibitor of metalloproteinase 1 (TIMP-1), and transforming growth factor β 1 (TGF- β 1) in cultured-activated HSCs treated with Cpd 861 or interferon- γ (IFN- γ) were determined by real-time PCR.

RESULTS: Both Cpd 861 and IFN- γ reduced the mRNA levels of collagen type III, MMP-2 and TGF- β 1. Moreover, Cpd 861 significantly enhanced the MMP-1 mRNA levels while down-regulated the TIMP-1 mRNA expression, increasing the ratio of MMP-1 to TIMP-1 to (6.3 + 0.3)-fold compared to the control group.

CONCLUSION: The anti-fibrosis function of Cpd 861 may be mediated by both decreased interstitial collagen synthesis by inhibiting the transcription of collagen type III and TGF- β 1 and increased degradation of these collagens by up-regulating MMP-1 and down-regulating TIMP-1 mRNA levels.

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Key words: Herbal Compound 861; Human hepatic

INTRODUCTION

Hepatic fibrosis, a wound-healing response to a variety of chronic liver injuries, is characterized by the increased deposition of remodeled extracellular matrix (ECM). Hepatic stellate cells (HSCs), the major source of ECM in liver, play a central role in the progress of fibrogenesis^[1-4]. After acute or chronic injury, HSCs are activated by autocrine and paracrine mediators and have greater fibrogenic, contractile and migration activities than “resting hepatic stellate cells” accompanied with expressing activation markers (e.g., α -smooth muscle actin, α -SMA). In addition to the synthesis of greater amounts of ECM components (predominantly collagen types I and III), activated HSCs also show altered expression/activity of matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs), thereby leading to increased deposition of extracellular matrix and formation of scar tissue in the fibrotic liver^[6-8]. Thus, the activated HSCs are considered a therapeutic target for antifibrotic drugs^[2-5].

Herbal compound 861 (Cpd 861) is an extract of mixed Chinese herbs that have been used for liver disease treatment in traditional Chinese medicine (TCM). Randomized double-blinded clinical studies have verified that Cpd 861 could significantly improve clinical manifestations and biochemical parameters of chronic HBV-related liver fibrosis patients, as well as regression of hepatic fibrotic change^[9-11]. Our preliminary data show that Cpd 861 could inhibit HSC proliferation and reduce the expression of α -SMA in culture-activated HSCs^[12]. The aim of the present study was

to identify the role of Cpd 861 in the regulation of collagen synthesis and degradation in culture-activated HSCs and to define its antifibrosis molecular mechanisms of action on the expression of collagens, MMPs, and TIMP-1 in human hepatic stellate cells.

MATERIALS AND METHODS

Subjects

Human hepatic stellate cell line LX-2 used in this study was kindly provided by Dr. Friedman SL of the Mount Sinai School of Medicine. The cells express α -SMA under all culture conditions and therefore are regarded as at least partially activated even after immediate replating^[13]. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, Invitrogen, Carlsbad, CA), supplemented with 5% heat-inactivated fetal bovine serum (FBS; Hyclone, USA), 200 mmol/L L-glutamine (Gibco, Invitrogen, Carlsbad, CA), 100 000 U/L penicillin (Sigma, USA) and 0.1 g/L streptomycin (Gibco, Invitrogen, Carlsbad, CA) in an atmosphere containing 50 mL/L CO₂.

Compound treatments

Cells were cultured in DMEM containing 5% FBS in an atmosphere containing 50 mL/L CO₂. The medium was carefully removed before compound treatment. The fresh medium containing Cpd 861 (0.01 g/L, in this concentration Cpd 861 had no effect on the proliferation of LX-2 cells)^[12] and IFN- γ (1 000 000 U/L, Fosun Pharma, Shanghai, China) was added. IFN- γ , which can reduce HSC activation both *in vivo* and *in vitro* associated with reduced extracellular matrix deposition in animal hepatic fibrosis progression^[14-17], was used as the positive control. The changes in mRNA expression of collagen types I and III, matrix metalloproteinase 1 (MMP-1), matrix metalloproteinase 2 (MMP-2), membrane type-1 matrix metalloproteinase (MT1-MMP), tissue inhibitor of metalloproteinase 1 (TIMP-1), and transforming growth factor β 1 (TGF- β 1) were detected by quantitative real-time PCR.

RNA isolation and reverse transcription

Total RNA was extracted from LX-2 cells as previously described^[12]. The concentration of total RNA was determined spectrophotometrically with ND-1000 spectrophotometer (NanoDrop, USA) and the integrity of samples was confirmed by visualizing 28 S and 18 S ribosomal RNA bands under ultraviolet light after agarose gel electrophoresis. One μ g of RNA was added to each reaction tube and converted to complementary DNA (cDNA) using oligo (dT)₁₅ primers (Promega, USA) and SuperScript™ II reverse transcriptase (Invitrogen, CA).

Quantitative real-time PCR using SYBR green I

Real-time PCR reaction was performed (10 min at 95°C for activation, 15 s at 95°C and 60 s at 60°C for 40 cycles of amplification) on the Applied Biosystems 7300 Real Time PCR System using SYBR® Green PCR Master Mix (Applied Biosystems, USA). Standard curve method and/or comparative CT method were used to quantify

Table 1 Oligonucleotide PCR primers for human genes

Oligonucleotide	Oligonucleotide primer	Fragment sequence size (bp)
Col I	L: GTCGAGGGCCAAGACGAAG R: CAGATCACGTCATCGCACAAAC	143
Col III	L: TGGTCCCAAGGTGTCAAAG R: GGGGGTCTGGGTACCATT	117
MMP-1	L: TCTGGGGTGTGGTGTCTCA R: GCCTCCCATCATTCTTCAGGTT	114
MMP-2	L: ACATCAAGGGCATTCAAGAG R: GCCTCCGTATACCGCATCAAT	268
MT1-MMP	R: GAAGCCTGGCTACAGCAATATG L: TGCAAGCCGTAAACTTCTGTC	119
TIMP-1	L: CTTCTGCAATTCCGACCTCGT R: CCCTAAGGCTTGGAAACCCTTT	127
TGF- β 1	L: GGCCAGATCCTGTCCAAGC R: GTGGGTTTCCACCATTAGCAC	201
GAPDH	L: ATGGGGAAGGTGAAGGTCG R: GGGGTCATTGATGGCAACAATA	108

the mRNA expression levels^[18,19]. Standard curves were generated using 10³-10⁹ copies of plasmids containing cDNA of each target gene, and results were normalized for RNA input using glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Samples and standards were analyzed in triplicate for each set of primers (Table 1).

After PCR, the melting curves of all final PCR products were analyzed as previously described^[12]. To ensure that the correct products were amplified in the reaction, all samples were also separated on 2% agarose gel electrophoresis. All PCR conditions and primers were optimized to produce a single product of the correct basepair size.

Statistical analysis

Data were expressed as mean \pm SE. Statistical analysis was performed using GraphPad Prism software (version 3.0). *t*-test was used for comparison between the groups. *P* < 0.05 was considered statistically significant.

RESULTS

Cpd 861 regulated the mRNA expression of hepatic fibrosis related genes.

To detect the effects of Cpd 861 on collagen synthesis and degradation in LX-2 cells, the mRNA levels of collagen types I and III, MMP-1, MMP-2, MT1-MMP, TIMP-1, and TGF- β 1 in cultured-activated HSCs after 48 h of treatment with Cpd 861 (0.01 g/L) or interferon- γ (1 000 000 U/L) were determined by real-time PCR. The data were normalized by GAPDH. As shown in Figure 1, both Cpd 861 and interferon- γ reduced the mRNA levels of collagen type III, MMP-2 and TGF- β 1. Moreover, Cpd 861, but not interferon- γ , significantly enhanced the MMP-1 mRNA levels, but at the same time inhibited the TIMP-1 mRNA expression which increased the ratio of MMP-1 to TIMP-1 to (6.3 \pm 0.3)-fold compared to the control group. In our study, the mRNA levels of collagen type I and MT1-MMP in LX-2 cells did not change much after 48 h of treatment with Cpd 861. To further understand the mechanisms of Cpd 861, the change over time was also detected. As shown in Figure 2, after 24 h of

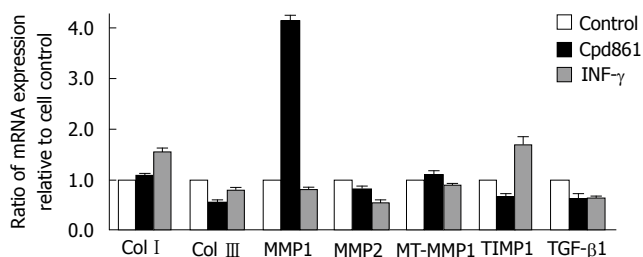


Figure 1 mRNA expression changes of collagen types I and III, MMP-1, MMP-2, TIMP-1, and TGF-β1 in LX-2 cells after 48 h of treatment with Cpd 861 (0.01 g/L) or interferon-γ (1 000 000 U/L). The levels of mRNA were determined by real-time PCR and normalized by GAPDH. Results are expressed as fold inductions compared to each control group. The data represent mean ± SE of triplicate for each treatment.

treatment with Cpd 861, the MMP-1 mRNA expression progressively increased significantly, while the expression levels of collagen type III, MMP-2, and TIMP-1 decreased significantly after exposed to Cpd 861 for 48 h or 72 h.

DISCUSSION

The altered balance between the synthesis and degradation of matrix proteins is the major pathogenic feature in the hepatic fibrosis process, which leads to the quantitative and qualitative change of composition in hepatic ECM. In advanced liver fibrosis, the total components of ECM increase accompanying a decrease in the normal low density basement membrane-like matrix (collagen type IV) being replaced by interstitial type matrix (mainly collagen types I and type III), leading eventually to the net deposition of fibrillar matrix^[20].

The extracellular matrix synthesis is mediated at both transcriptional and post-transcriptional levels, while its degradation is predominantly regulated by MMPs, which can degrade almost all ECM components. For example, MMP-2 (gelatinase A), one of the major MMPs during liver fibrogenesis, degrades type IV collagen, and this function can enhance the normal basement replacement by fibril-forming collagen which contributes to the pathogenesis of liver fibrosis. MMP-2 is secreted as a pro-enzyme, and its activation involves a membrane type MMP (MT1-MMP) expressed on the cell surface^[21,22]. In contrast to MMP-2, interstitial collagenases (MMP-1 in humans, MMP-13 in rats) are involved in the degradation of interstitial type matrix by cleaving such substrates at a specific Gly-Ile/Leu site^[6]. The activities of MMPs are inhibited by TIMPs (a family of tissue inhibitors of metalloproteinases, TIMP-1 and TIMP-2 especially in the liver)^[6]. The combination of MMPs and TIMPs plays a critical role in the remodeling of hepatic ECM during liver fibrogenesis. When liver fibrosis progresses, in addition to increased matrix synthesis, the enhanced expression and release of MMP-2 and MT1-MMP as well as TIMP-1 are also the striking features, while the expression of MMP-1 decreases or remains unchanged, which serves to promote progression of liver fibrosis by preventing degradation of interstitial collagens^[6,23].

In the liver, HSCs are the key cell type involved both in the synthesis and in the degradation of matrix

proteins. These cells are the major fibrogenic cell type that contributes to collagen accumulation in the liver. In a normal liver, HSCs in the space of Disse are maintained in a quiescent, nonfibrogenic phenotype. When the liver is injured, these cells are activated and transformed to myofibroblast-like cells characterized by fibrogenesis, contractility, and migration, production of much larger amounts of the majority components of extracellular matrix, particularly collagen types II and III. Moreover, satellite cells also express almost all the key components required for matrix degradation such as MMP-1, MMP-2 and TIMP-1^[1,24]. Changes in the activity of MMPs and TIMPs lead to the remodeling of hepatic ECM during injury to the liver, which in turn directly and indirectly accelerates stellate cell activation.

LX-2 cells, a human hepatic stellate cell line, were used in our study. LX-2 cells retain features of HSCs and express key proteins involved in matrix remodelling^[13]. The cells express α-SMA under all culture conditions and are regarded as, at least, partially activated even after immediate replating. Furthermore, during growth on a plastic surface, the cells undergo further activation as defined by the increase in α-SMA^[25]. In our study, the collagen type III and MMP-2 mRNA expression increased, while MMP-1 mRNA expression was down-regulated when these cells were further activated on a plastic surface (data not shown), consistent with those seen in clinical or animal studies^[6,23]. Thus, LX-2 cells provide a valuable tool in our anti-fibrotic drug research.

Cpd 861 formulated by one of the authors (Bao'en Wang)^[10] according to Chinese medical theory, is comprised of *Salvia miltiorrhiza*, *Astragalus membranaceus* and *Spatholobus suberectus* as its chief components. This herbal compound has been proven to be effective for the treatment of patients with hepatic fibrosis and its therapeutic effectiveness in making hepatic fibrosis regress has been confirmed by several random clinical tests with paired liver biopsies and animal model studies^[10,11,26,27]. Our previous study demonstrated that Cpd 861 can significantly inhibit cell proliferation in a dose-dependent manner and reduce the mRNA expression level of α-SMA in LX-2 cells^[12]. In this study, we used real-time PCR to identify mRNA expressions of collagens, MMPs, and TIMPs in culture-activated HSCs. The data show both Cpd 861 and positive control IFN-γ inhibited the mRNA expressions of TGF-β1, MMP-2, and collagen type III, while there was no difference in the mRNA expression of MT1-MMP and collagen type I observed in LX-2 cells treated with either Cpd 861 or IFN-γ alone.

As the dominant stimulus of ECM production by HSCs, the increasing expression of TGF-β1 has been demonstrated in both culture-activated HSCs and *in vivo* studies, and the autocrine expression in activated HSCs is its most important resource^[28-30]. Besides direct transcriptional down-regulation of collagen type III, the restraining of mRNA expression of TGF-β1 may also play an important role in both Cpd 861 and IFN-γ anti-collagen-synthesis functions.

As discussed above, the degradation of basement membrane by HSC-derived MMP-2 is critical to further activation of HSCs and scar formation during liver

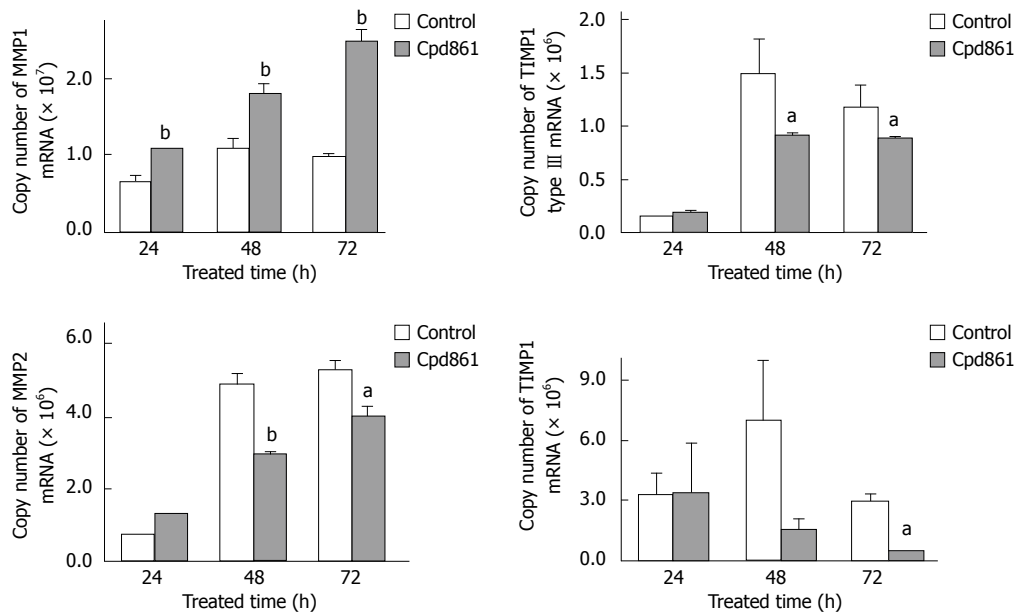


Figure 2 Time course of Cpd 861 on mRNA expressions in LX-2 cells. LX-2 cells were incubated with Cpd 861 (0.01 mg/mL) for 24, 48 and 72 h. The absolute copy number of mRNA for collagen type III, MMP-1, MMP-2, and TIMP-1 was determined by real-time PCR. The data represent mean \pm SE of triplicate for each treatment. ^a $P < 0.05$ and ^b $P < 0.01$ vs LX-2 cell controls.

wound remodeling. The transcriptional down-regulation of MMP-2 by both Cpd 861 and IFN- γ may protect the normal basement membrane from being replaced by fibrillar matrix and indirectly inhibit the activation of HSCs.

It is particularly interesting that, in contrast to IFN- γ , Cpd 861 also can significantly enhance the mRNA level of MMP-1 and decrease the TIMP-1 mRNA expression, indicating that Cpd 861 can directly (enhancing the expression of MMP-1) and indirectly (inhibiting the expression of TIMP-1) enhance the degradation of collagens. The data from the time course study indicate that the up-regulation of MMP-1 expression preceded the changes in MMP-2, TIMP-1, and collagen type III mRNA levels, suggesting that the fibrillar matrix degradation has already enhanced before its synthesis is inhibited by Cpd 861. In addition to their role in inhibiting extracellular matrix degradation, there is increasingly recognized that TIMPs play a significant role in regulating apoptosis of some cell types^[6]. Thus, the down-regulation of TIMP-1 mRNA levels by Cpd 861 may also contribute to pro-apoptosis function in the HSCs due to its mechanism underlying anti-fibrosis.

In conclusion, the anti-fibrotic function of Cpd 861 may be attributed to both the decreased interstitial collagen synthesis by down-regulating the mRNA levels of collagen type III and TGF- β 1 as well as increased degradation of these collagens by up-regulating MMP-1 and down-regulating TIMP-1, accompanying protection of the normal basement membrane from destruction by MMP-2, and perhaps at some points associated with the apoptosis of activated HSCs. Further work is required to accurately analyze the relative roles of these different functions in detail, and this will be important in the development of novel antifibrotic therapies.

ACKNOWLEDGMENTS

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COMMENTS

Background

The altered balance between the synthesis and degradation of matrix proteins is the major pathogenic feature in the hepatic fibrosis process. The purpose of this study was to identify the role of herbal compound 861 (Cpd 861) in the regulation of mRNA expression of collagen synthesis- and degradation-related genes in human hepatic stellate cells (HSCs).

Research frontiers

Although remarkable progress has been made in understanding the mechanisms underlying hepatic fibrosis and plenty of agents have been studied, few effective "anti-fibrogenic" drugs have been approved for use in humans.

Innovations and breakthroughs

The study not only verified the clinical anti-fibrotic effect of the herbs, but also explored the multiple action sites of the herbal compound which is evidently different from that of the purified ingredients in Western drugs.

Applications

The results of this study could allow for developing the effective anti-fibrogenic drugs from the Chinese herbs.

Peer review

This is an interesting paper, describing the effect of plant extract Cpd 861 on the levels of mRNAs encoding collagens (types I and III, metalloproteinases (MMP-1 and MMP-2), and various other factors (TIMP-1 and TGF- β 1) in LX-2 cells of stellate cell origin. The plant extract appeared to regulate the mRNA levels of collagen synthesis- and degradation-related genes, thus demonstrating an anti-fibrotic effect.

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Perforated appendicitis masquerading as acute pancreatitis in a morbidly obese patient

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Abstract

Diagnosis and treatment of common conditions in morbidly obese patients still pose a challenge to physicians and surgeons. Sometimes too much reliance is put on investigations that can lead to a misdiagnosis. This case demonstrates an obese woman admitted under the medical team with a presumed diagnosis of pneumonia, who was later found to have an acute abdomen and raised amylase, which led to an assumed diagnosis of pancreatitis. She died within 24 h of admission and post mortem confirmed the cause of death as systemic sepsis due to perforated appendicitis, with no evidence of pancreatitis. Significantly elevated serum amylase level may occur in non-pancreatic acute abdomen.

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Key words: Morbid obesity; Perforated appendicitis; Pneumonia; Serum amylase

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INTRODUCTION

Obesity is an increasing problem in the western population and the proportion of morbidly obese people who present with non-obesity problems is increasing. An important

consideration in dealing with such patients is the difficulty of diagnosis. We present a patient with acute abdomen and morbid obesity and discuss the unusual finding of markedly elevated serum amylase due to a non-pancreatic cause.

CASE REPORT

The patient (AE) was a morbidly obese (BMI 59) 60-year-old woman with type 2 diabetes, asthma and previously diagnosed gallstones, who was referred by her general practitioner to hospital with breathlessness and a provisional diagnosis of pneumonia. Upon admission, she complained of abdominal pain, which was worse on coughing. She had pyrexia (39.4°C) tachycardia (pulse 125/min) and hypotension (90 mmHg systolic blood pressure). There were fine crepitations in both lung fields and vague right upper abdominal tenderness. A chest X-ray was requested and she was transferred to a respiratory ward.

AE was seen 3 h later by a medical Senior House Officer who thought the chest X-ray was normal, although this was reported later by a radiologist to show diffuse infiltrative shadowing consistent with acute respiratory distress syndrome (ARDS). Investigations revealed renal impairment, normal bilirubin, but raised plasma liver enzyme levels, a serum amylase level of 1029 IU/L and neutrophilia. AE was started on antibiotics as she was presumed to have acute pancreatitis and referred to the surgical team.

AE was assessed by a surgical registrar 8 h after her admission and was found to be systemically unwell, with a systolic blood pressure of 100 mmHg, pulse of 120/min, and pyrexia of 39°C. Further history taking revealed generalized abdominal pain of 5 d duration. Abdominal examination was deemed ineffective to elicit meaningful signs, due to extreme truncal obesity. The diagnosis of pancreatitis was confirmed and because of her progressive deterioration, resuscitative measures were commenced and she was referred to the High Dependency Unit (HDU). In the HDU, she had non-invasive positive pressure ventilation, invasive monitoring, and measures adopted to achieve glycemic control.

On the post take surgical ward round, the consultant on call confirmed the difficulty and unreliability of abdominal examination in such a morbidly obese patient, but given the diagnosis of acute pancreatitis, he/she advised continuation of supportive care. Shortly after AE was reviewed, she suffered a cardiac arrest and,

despite resuscitation, died. The post mortem confirmed death from generalized peritonitis and septicemia due to ruptured appendicitis. The pancreas was normal with no evidence of inflammation.

DISCUSSION

This case highlights the difficulty in making accurate diagnosis in a morbidly obese patient. It is entirely understandable why a patient referred by her general practitioner with a provisional diagnosis of pneumonia, and who upon general examination had a vague upper abdominal tenderness would be presumed to have just a chest infection. Whether the ARDS picture on her chest X-ray was missed due to extreme obesity or not is unclear. Routine blood analysis in this patient showed deranged liver function tests which necessitated the request for serum amylase analysis. Given the high level of serum amylase, it was reasonable to make a diagnosis of acute pancreatitis. However, further enquiries revealed AE had had pain for > 5 d. In retrospect, this should have raised doubt about the diagnostic value of the elevated serum amylase level. It would appear there was an over reliance on the serum amylase result by all the teams involved in her care. Serum amylase can be raised in many acute peritonitis conditions, such as perforated duodenal ulcer, appendicitis^[1] or small bowel ischemia^[2]. What makes this case unique are the history of gallstones and an excessively high level of serum amylase- both of which contributed to the misdiagnosis. Furthermore, as indicated above, serum amylase usually falls within the first 48 h of the onset

of pancreatitis^[3]. Liver enzymes may also rise in sepsis^[4]. The main obstacle to a proper and thorough physical examination in this patient was her body habitus. Given the acknowledged difficulty with examination, there is a strong case for an even more detailed history and an early recourse to the use of ancillary investigations. A computed tomography (CT) scan of the abdomen in this case might have revealed a normal pancreas and the possibility of a potential life saving laparotomy. However, it is debatable whether emergency surgery in the presence of morbid obesity, medical comorbidities and systemic sepsis syndrome would have produced a different outcome. Another consideration is that this large patient might not have fitted into the CT scanner.

In conclusion, morbidly obese patients who present with acute medical or surgical problems require detailed and thorough history taking, coupled with early and appropriate investigations, in order to avoid diagnostic pitfalls. Significantly elevated serum amylase level may occur in the non-pancreatic acute abdomen.

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Hemobilia due to hepatic artery aneurysm as the presenting sign of fibro-muscular dysplasia

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Abstract

Fibro-muscular dysplasia (FMD) is a rare but well documented disease with multiple arterial aneurysms. The patients, usually women, present with various clinical manifestations according to the specific arteries that are affected. Typical findings are aneurysmatic dilations of medium-sized arteries. The renal and the internal carotid arteries are most frequently affected, but other anatomical sites might be affected too. The typical angiographic picture is that of a "string of beads". Common histological features are additionally described. Here we present a case of a 47-year-old woman, who was hospitalized due to intractable abdominal pain. A routine work-up revealed a liver mass near the portal vein. Before a definite diagnosis was reached, the patient developed massive upper gastrointestinal bleeding. In order to control the hemorrhage, celiac angiography was performed revealing features of FMD in several arteries, including large aneurysms of the hepatic artery. Active bleeding from one of these aneurysms into the biliary tree indicated selective embolization of the hepatic artery. The immediate results were satisfactory, and the 5 years follow-up revealed absence of any clinical symptoms.

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Key words: Fibro-muscular dysplasia; Hemobilia; Endovascular approach

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CASE REPORT

A 47-year-old woman was admitted to the emergency department (ED) with epigastric abdominal pain, nausea, and vomiting for 2 d. The patient denied fever or changes of bowel habits. The pain did not subside following a medication with antacids and H2 blockers prior to admission.

Physical examination revealed a soft abdomen with epigastric tenderness and the absence of jaundice and peritoneal irritation signs. It revealed no further findings.

Upon admission, white blood count, liver function test, bilirubin levels, and amylase activity were within the normal range. Abdominal X-ray was within normal limits. Abdominal ultrasound (US) demonstrated a cystic structure near the hepatic hilum. Gastro-duodenoscopy was performed without revealing any pathology. An abdominal CT scan demonstrated a soft tissue mass encapsulating the portal vein and hepatic artery causing intra-hepatic biliary tree dilatation (Figure 1A). Following an increase of the total bilirubin level of up to 0.39 g/L, endoscopic retrograde cholangio-pancreaticography (ERCP) was performed. ERCP revealed a common hepatic duct stricture with proximal biliary dilatation (Figure 1B). A stent was inserted without complications. Biliary brush biopsy was taken and turned out to be normal. With the working diagnosis of a neoplastic process, endoscopic ultrasound (EUS) guided biopsy was scheduled. While awaiting the procedure, the patient's condition stabilized, the pain resolved, and she was discharged. A few days later she presented with complaints of severe abdominal pain accompanied by jaundice and hematemesis. Upon arrival to the ED her pulse was non-palpable and systolic blood pressure was 70 mmHg. After aggressive fluid resuscitation and blood transfusion emergent gastro-duodenoscopy was performed, once again without any pathologic finding. Abdominal US revealed the described soft tissue mass which had doubled in size and appeared to be pulsating. With the working diagnosis that the tissue mass was of vascular origin, angiography was carried out. The hepatic, renal, and iliac arteries had a radiographic appearance of a

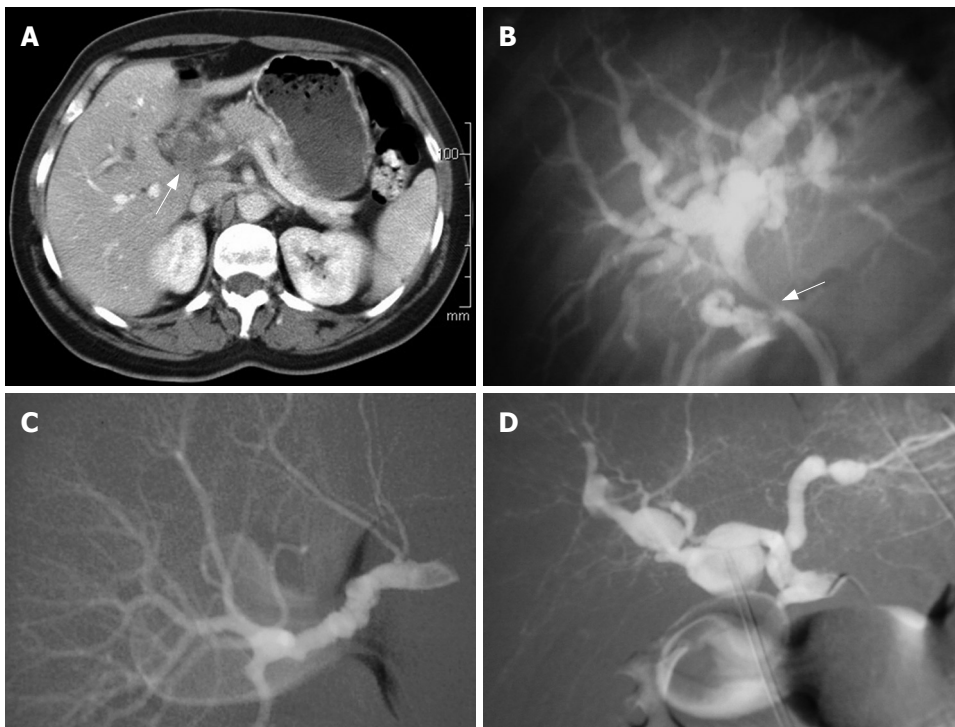


Figure 1 Clinical examination results. **A:** Abdominal CT scan showing a soft tissue mass encapsulating the portal vein and hepatic artery (arrow); **B:** ERCP showing a common hepatic duct stricture (arrow) with proximal biliary tree dilatation; **C:** Selective angiography of the renal artery demonstrating the classic appearance of "string of beads"; **D:** Selective angiography of the hepatic artery revealing multiple aneurysms.

"string of beads" (Figure 1C and D), which characterizes fibro-muscular dysplasia (FMD). In order to prevent further bleeding from hepatic artery aneurysms to the biliary system, embolization of the proper hepatic artery using a thrombogenic coil was performed. Elevated levels of bilirubin indicated concurrent ERCP which revealed a clogged stent, obstructed by blood clots, and which thus was replaced. After the procedure the patient did not suffer from gastrointestinal bleeding, and elevated liver enzyme levels gradually resolved. The patient was discharged 52 d after her first admission.

Today, 5 years after the first symptoms, the patient has no clinical signs or symptoms related to hepatic artery embolization, and no laboratory or radiographic changes are evident.

DISCUSSION

FMD is a disorder which leads to arterial stenosis and most commonly affects the renal and internal carotid arteries in 60%-75% and 25%-30% of cases, respectively^[1,2]. It is double as common among females and most cases are diagnosed in patients younger than 50 years.

The pathogenesis is not well understood and might include a genetic predisposition, hormonal influences, mechanical factors, or ischemia. All of the above mentioned parameters might contribute to deposition of fibrous lesions, which are classified into five categories according to the affected arterial layer and the histological pattern^[3].

Clinical manifestations result from ischemia (due to arterial stenosis), hemorrhage (due to ruptured aneurysms), and embolization of intravascular thrombi formed within the aneurysms. Thus, they may vary widely depending on the arteries involved^[4].

A common manifestation of FMD is reno-vascular

hypertension due to renal artery stenosis. The clinical presentation of an affected carotid artery may vary from headache and lightheadedness to cerebrovascular accident.

The gold standard imaging technique for the diagnosis of FMD is angiography, which demonstrates several features such as beading of the vessel and concentric stenosis. The classical appearance in the most common of the five pathological subtypes is a "string of beads"^[2]. Other diagnostic modalities include duplex-ultrasound, CT-angiography, and MR-angiography^[5].

Treatment of FMD depends upon the arteries involved and the clinical presentation. In cases of regional ischemia, revascularization (by either endovascular or surgical approach) is the preferred treatment. Hemorrhagic complications are treated by ligation or embolization of the affected artery. Hemobilia is a rare clinical condition which may be fatal if not treated promptly. It was first described in 1948 following abdominal trauma^[6]. Since then, many patients suffering from hemobilia were described, most of them were treated surgically^[7]. Endovascular embolization of ruptured hepatic artery aneurysms have been described^[8,9] but not in the context of FMD.

FMD of the hepatic artery is only one of many etiologies that might cause hemobilia and it mandates treatment even if asymptomatic^[10]. Only one case in which FMD was the underlying cause for hemobilia was described in the English literature^[11]. That patient was treated surgically by ligation of the common hepatic and gastroduodenal arteries. The patient we report was the first who was treated with a minimally invasive endovascular technique for FMD related hemobilia.

In some cases of controllable gastrointestinal bleeding due to FMD related hemobilia, if the patient's hemodynamic status permits, selective angiography and embolization of ruptured hepatic artery aneurysms may

be highly effective. The endovascular approach holds less morbidity and mortality than surgery and serves both as a diagnostic and a therapeutic tool. Success in treating such a patient is dependent upon good communication and cooperation between the surgeon and the interventional radiologist.

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Events Calendar 2008-2009

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 EASL-AASLD-APASL-ALEH-IASL Conference Hepatitis B and C virus resistance to antiviral therapies
www.easl.ch/hepatitis-conference

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

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

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

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

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

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

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

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

April 23-27, Milan, Italy
 43rd Annual Meeting of the European

Association for the Study of the Liver
www.easl.ch

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

May 18-21, San Diego, California, USA
 Digestive Disease Week 2008
 June 4-7, Helsinki, Finland

The 39th Nordic Meeting of Gastroenterology
www.congex.com/ngc2008
 June 6-8, Prague, Czech Republic
 3rd Annual European Meeting: Perspectives in Inflammatory Bowel Diseases
 Email: meetings@imedex.com

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

June 25-28, Barcelona, Spain
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 Imedex and ESMO
 Email: meetings@imedex.com

June 25-28, Lodz, Poland
 Joint Meeting of the European Pancreatic Club (EPC) and the International Association of Pancreatology (IAP)
 E-mail: office@epc-iap2008.org
www.e-p-c.org
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June 26-28, Bratislava, Slovakia
 5th Central European Gastroenterology Meeting
www.ceurgem2008.cz
 September 10-13, Budapest, Hungary

11th World Congress of the International Society for Diseases of the Esophagus
 Email: isde@isde.net

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

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 Falk Workshop: Strategies of Cancer Prevention in Gastroenterology

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 Falk Symposium 166: GI Endoscopy - Standards & Innovations

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

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

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



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 18th World Congress of the International Association of Surgeons, Gastroenterologists and Oncologists
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www.negf.org
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 59th AASLD Annual Meeting and Postgraduate Course
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- 3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

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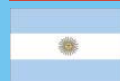
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^[1]Passed away on October 20, 2007

^[2]Passed away on June 11, 2007

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Intragastric injection of botulinum toxin for the treatment of obesity. Where are we?

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Abstract

Obesity has reached epidemic proportions particularly in western countries. Most non-surgical treatments of this condition are disappointing. Since 2005, several studies evaluating the effect of Botulinum Toxin type A (BT-A) in gastric antrum by means of endoscopy for the treatment of obesity have been published. This treatment modality was based on the observation that gastric injection of BT-A in laparatomized rats induced a significant reduction of food intake and body weight. Nowadays, 6 studies have been published yielding conflicting results. Differences in selection of patients, doses of BT-A, method of administration of the toxin and instruments of evaluation of some parameters among these studies may be the cause of divergent results. We discuss herein some important features of these studies pointing out on differences among them. At the same time, based on the knowledge of physiological characteristics of normal and abnormal gastric function related with feeding, we discuss the probable causes of failure observed in these trials. Finally, we give some guidelines concerning the way that future research in this field may follow, not without calling attention to disadvantages of this treatment.

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Key words: Botulinum toxin; Obesity; Gastric emptying; Gastric motility; Gastroparesis

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INTRODUCTION

The prevalence of obesity has increased in western countries in the last few decades, reaching epidemic proportions^[1]. It affects more than 30% of general population in the US. In this country, the costs attributed to obesity amounts to 100 billion dollars per year^[2] and the number of deaths attributed to obesity is approximately 280 000 annually^[3]. Obesity increases the risk of morbidity and mortality, since some disorders such as diabetes, arterial hypertension, cardiovascular and cerebral illnesses, as well as hepatobiliary disorders, are particularly frequent in obese individuals^[2].

The dietetic, pharmacological and behavioral treatments have demonstrated to have limited effect and duration^[4]. The intragastric balloon applied by endoscopy has equally given partial and transitory results^[5]. Surgical treatments (gastric banding and gastric by-pass), even if they are the most effective in some patients, particularly those with morbid obesity, are invasive procedures and may have complications, some of them fatal^[6]. In view of the above, the search for new methods for weight reduction is completely justified.

In the year 2000, Gui *et al* published a pioneering study in which they show that intra-muscular injections of Botulinum Toxin type A (BT-A) in the gastric wall of laparatomized normal-weight rats significantly reduced their food intake and body weight^[7]. Subsequently, such findings were confirmed in 2005, by Coskun *et al* in obese rats. This group also observed a significant delay of gastric emptying in rats that had received BT-A^[8]; therefore, they attributed body weight reduction to an effect of early satiety probably induced by the pharmacologically induced gastroparesis.

BOTULINUM TOXIN

Botulinum Toxin is produced by the bacterium *Clostridium botulinum*. There are several serotypes from A to G. When this toxin is ingested by the human being it can produce a form of food poisoning known as botulism. The BT-A

has a powerful inhibiting effect of long duration on the muscular contractions of smooth and striated muscles^[9]. This pharmacological property has been used in the treatment of some digestive illnesses characterized by muscular spasm, particularly achalasia and anal fissure^[10,11]. BT-A binds with high affinity to cholinergic nerve endings and selectively inhibits their activity. Acetylcholine is considered the most important stimulating agent both in intrinsic (myenteric) and extrinsic (vagal) nervous systems^[12].

SATIETY AND GASTRIC MOTILITY

Additionally, the mechanisms that induce the gastric satiety are complex and they are related to the motor function of the stomach as well as to endocrine and paracrine effects acting in interrelated form. It is known that several mechanisms are involved in the induction of satiety such as distension and accommodation of the stomach, as well as hormones such as cholecystokinin (CCK), glucagon-like peptide (GLP-1), bombesin, liberating-gastrin peptide and somatostatin. It has also been observed that ghrelin, which is a peptide produced in the stomach, has orexigenic effect that probably controls the appetite at a central hypothalamic level. Other factors also intervene for the control of appetite as glycemia and some hormones such as insulin, leptin and enterostatin. It has been observed, for example, that duodenal infusion of fat induces a delay of gastric emptying and sensation of satiety^[13]. Additionally, gastric banding increases the cholecystokinin plasma levels^[14], the Roux-en Y gastric by-pass inhibits basal and postprandial ghrelin plasma levels and increases peptide YY (PYY) concentrations^[15]. The jejunoileal by-pass increases cholecystokinin, motilin, GLP-1 and PYY^[16], delays gastric emptying and reduces hunger sensation. As cholecystokinin, ghrelin and PYY also influence the gastrointestinal motility, it may be possible that a mechanism related to modifications of the gastric emptying is responsible for the early satiety and reduction of body weight observed in these operated patients.

Also the patterns of the gastric motility are well known. The fundus and proximal portion of the gastric body relax during the prandial and postprandial period; therefore, the intra gastric pressure is not modified in a significant form at the beginning of food ingestion. This phenomenon is known as "gastric accommodation", a term which was introduced almost 100 years ago^[17]. It consists of a receptive relaxation induced by the bolus deglutition and an adaptive relaxation influenced by the increase of the intragastric pressure due to food accumulation into the stomach. The impairment of the gastric accommodation seems to be initially responsible for the sensation of fullness and satiety^[18]. Meanwhile, gastric antrum muscles contract in concentric form by means of rings of distal displacement impelling the gastric content to the duodenum. Nevertheless, the pylorus in postprandial period contracts preventing the early passage of solid meals to the duodenum. Thus, meals are returned to the gastric body in repeated form^[19]. The speed with which the stomach empties depends on the nature of meals (the solids retain more time than the liquids), of the

osmolality (the isosmotic meals retain less time than the hypo-osmotic and hyper-osmotic) and of the chemical composition (the fats retain the most time). The hormonal mediators previously mentioned are produced by means of chemical and mechanic stimuli triggered by meals in the stomach and the proximal intestine and their main function is regulation of the gastrointestinal motility.

GASTROPARESIS

Gastroparesis is a gastric disorder characterized by a delay in the gastric emptying. The etiology is very diverse. The typical clinical manifestations are eructation, early satiety and sensation of gastric fullness, epigastric discomfort, nausea and vomiting and reduction of body weight^[20]. It has been found that the patients with anorexia nervosa have a significant delay of gastric emptying compared to normal individuals or those with bulimia^[21].

CLINICAL STUDIES OF BT-A FOR TREATMENT OF OBESITY

In accordance with all mentioned above, the clinical use of the BT-A injected into the gastric antrum in obese patients for inducing gastric emptying delay and body weight reduction seemed logical.

This idea was reinforced from the report of Rollnik *et al*, of a patient in whom the injection of BT-A in the gastric antrum by endoscopy was associated with a reduction of 9 kg of body weight and 32.5% of the caloric daily intake 4 mo after treatment^[22].

In the last two years, 6 studies evaluating this novel treatment have been published^[23-28]. Three were open pilot and 3 were randomized double blind controlled trials (one of them performed by our group^[23]) of which in only one, beneficial effect of BT-A on body weight reduction was observed^[27]. Nevertheless, important differences among these studies deserve to be discussed in detail (Table 1).

The dose of BT-A

The dose of BT-A used in all the studies was highly variable. It ranged from 100 UI to 300 UI. However, in the study in which the maximum dose was used no effect on body weight reduction was observed. Perhaps more important than the dose of BT-A was the method of application.

Method of application of BT-A

In all the studies, BT-A was administered by means of endoscopic antral injections in a number of punctures that ranged from 8 to 24 in circular disposition. Probably, it was expected that the more the punctures performed the more intra muscular diffusion of the toxin might have been obtained. Nevertheless, this factor was not crucial since in the study in which the greatest number of punctures was done, (24 punctures) the results were negative.

It is important to point out that BT-A were injected both into the antrum and the gastric fundus in the only study in which positive results were obtained (in the rest of the studies only antral injections were done). If we remember, the gastric fundus does not have a propulsive

Table 1 Description of the results of 6 studies in which intragastric injection of Botulinum Toxin type A was administered to obese patients for treatment of obesity

Reference	n	Design	Dose (UI)	Follow-up (wk)	Results
Garcia-Compean ^[23]	12	Pilot	100 antrum	12	Reduction of body weight: No Gastric emptying: Negative Reduction of body weight: No
Albani ^[24]	8	Pilot	100 antrum	16	Reduction of body weight: No
Cardoso ^[25]	12	Pilot	200/300 antrum	12	Early satiety: Yes Reduction of body weight: No Gastric emptying: Negative
Gui ^[26]	14	RCT ¹	133/200 <i>vs</i> saline antrum	8	Early satiety: Yes Reduction of body weight: No Gastric emptying: Negative
Foschi ^[27]	24	RCT ¹	200 <i>vs</i> saline antrum + fundus	8	Early satiety: Yes Reduction of weight: Yes Gastric emptying: Positive Max. gastric capacity for liquids: Positive
Mittermaier ^[28]	10	RCT	200 <i>vs</i> saline / antrum	24	Early satiety: No Reduction of weight: No

¹RCT: Randomized controlled trials.

effect as the antrum, injections in this place to cause gastric emptying delay would not seem to have justification. Notwithstanding, the existence of other mechanisms related to satiety that might have origin in the fundus must be considered as we will discuss later.

Early satiety

Of 4 studies in which early satiety was evaluated after therapy, a positive effect was observed in 3 (two of them were randomized double blind controlled trials). However, only in 1 of these 3 studies a significant body weight reduction was observed. This incongruousness between early satiety and absence of weight reduction observed in some studies may be due to the difficulties of measuring a subjective parameter like this, or perhaps the intensity of the early satiety was not enough to produce significant body weight loss.

Gastric emptying

In only 1 of 5 studies in which gastric emptying after therapy was evaluated a significant delay was observed. Notwithstanding, diverse methods were used for measuring this parameter: octanoic acid breath test, gastric emptying scintigraphy for solids and liquids labeled with Technetium 99 and Indium 111, respectively. It is well known that results of these procedures can be affected by several factors (type of test meals, chemical composition and osmolarity of the test meals, quantity of liquid, *etc.*). For this reason these procedures must be carefully standardized in every laboratory. In regards to the above mentioned, presently highly sensitive and specific procedures for measuring gastric emptying are not available^[29].

HOW TO EXPLAIN THE DIFFERENCES OF RESULTS BETWEEN THE ONLY POSITIVE AND THE 5 NEGATIVE STUDIES?

In the only positive study performed by the Italian group,

8 injections of BT-A were done in the gastric fundus in addition to the injections in gastric antrum. Conversely in the other studies, injections in the antrum were only done. In this positive study a significant modification of all the evaluated parameters were observed after treatment: presence of early satiety, a delay in gastric emptying, a reduction of the maximal gastric capacity for liquids and more importantly; a significant reduction of body weight. As authors of this study pointed out, gastric fundus is the principal source of ghrelin^[30] and it also has sensory activity that regulates the total gastric capacity^[31]. Ghrelin is a 28 amino acids peptide produced by the stomach with orexigenic effect acting on the arcuate nucleus of the hypothalamus. Ghrelin plasma levels increase during periods of fasting and reduce after a meal, in other words, this peptide seems to have a regulatory effect of hunger. However, published studies have shown that ghrelin expression in gastric mucosa, measured by histochemistry, increased one year after gastric banding in obese patients who maintained body weight loss; this would discard the physio-pathogenic role of ghrelin in body weight loss of these patients^[32]. Similarly, in another study, high ghrelin plasma levels did not predict a minor loss of body weight in patients with gastric banding compared to patients with normal plasma ghrelin levels^[33]. Conversely, Roux-en-Y gastric by-pass inhibits basal and postprandial ghrelin plasma levels^[15]. Additionally, ghrelin increases gastric emptying and stimulates gastric motility during fasting^[34]. For all the above mentioned, it is difficult to clarify the role of ghrelin in body weight reduction of the patients in the positive study, particularly when plasma levels of this peptide were not measured.

The reduction of the maximal capacity for liquids after BT-A treatment may be explained by impairment of the gastric fundus accommodation inducing early satiety. Nevertheless, the test of gastric maximal capacity for liquids has poor reproducibility for measuring gastric accommodation. Recently, a novel scintigraphic method for simultaneously assessing gastric accommodation and emptying has been developed using dual-isotopes,

either (^{99m}Tc -pertechnetate intravenously and (^{111}In -diethylenetriaminepentaacetic acid in a liquid nutrient drink or an (^{111}In -oxine-labeled egg sandwich meal. Emptying and accommodation were measured using single positron emission computer tomography (SPECT) every 20 min and up to 240 min^[35].

On the other side, the mean delay of gastric emptying observed in patients after BT-A, although significant, was short. Therefore, it makes it difficult to attribute early satiety and body weight reduction to this mechanism.

Finally, treated and untreated patients were given reductive diets of 1200 kcal/day. This may explain the reason why non treated patients also had a significant body weight reduction. Therefore, it is very probable that in treated patients a combined effect of reductive diet and toxin was observed.

FUTURE OF BT-A IN THE TREATMENT OF OBESITY

In the context of all the above discussed, the following question arises: What is the future of the endoscopic gastric injections of BT-A for the treatment of obesity?

In our opinion the method of antral injections has a very uncertain future. If we take into account that this drug is expensive (100 UI cost about 350 Euros or \$530 dollars), the performance of a study on a major scale is very difficult to achieve given the present circumstances.

Notwithstanding, it remains to be clarified if BT-A injections in the gastric fundus have better results in body weight reduction in obese patients. Perhaps the mechanism of action would be more difficult to explain. Modifications of gastric accommodation inducing early satiety may be an attractive hypothesis. Nevertheless, the measurement of this parameter in future studies by means of reliable tests will be the obstacle to overcome.

If gastric injections of BT-A demonstrate to be effective for the treatment of obese patients in the future, there is another disadvantage that must be considered: the limited duration of its effect (3 mo-6 mo). Therefore, for long-term administration by repeated administration of this drug, the cost-benefit relation has to be taken into account.

In medical science, it is frequent to find an agent that works and less frequent to know how it works. Consequently, we considerably learn from the test error method.

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OBSERVER

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Colorectal cancer risk in Crohn's disease

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Abstract

There is recognized increased risk for colorectal cancer in patients with inflammatory bowel disease, particularly in long-standing and extensive ulcerative colitis. There also appears to be an increased rate of intestinal cancer in Crohn's disease, including both colon and small bowel sites. In Crohn's disease, evidence suggests that detection of colorectal cancer may be delayed with a worse prognosis. Some risk factors for cancer in Crohn's disease include the extent of inflammatory change within the colon and the presence of bypassed or excluded segments, including rectal "stump" cancer. In addition, the risk for other types of intestinal neoplasms may be increased in Crohn's disease, including lymphoma and carcinoid tumors. Earlier detection of colorectal cancer based on colonoscopy screening and surveillance may be achieved but, to date, this has not translated into a positive survival benefit. Moreover, newer staining methods and evolving micro-endoscopic techniques show promise, but have not significantly altered management. Future research should focus on development of molecular or other bio-markers that might predict future dysplasia or cancer development in Crohn's disease.

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Key words: Colon cancer; Crohn's disease; Surveillance; Small bowel cancer

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INTRODUCTION

Previous studies documented that patients with inflammatory bowel disease, particularly those with extensive and long-standing ulcerative colitis, have an increased risk of later colorectal cancer development. This data, however, was largely based on investigations conducted in tertiary care settings, especially from the United States and the United Kingdom. Later studies, particularly from similar geographic locations in the United States, demonstrated that the magnitude of this increased risk may not be so significant in a private or community practice setting^[1,2]. In contrast, others have suggested that the risk of colorectal cancer in patients with colitis is not universally increased^[3]. In part, this may be influenced by the underlying colorectal cancer risk related to individual inherited, geographic or other environmental factors, rather than inflammatory bowel disease *per se*.

CROHN'S DISEASE AND CANCER RISK

In Crohn's disease, specifically, precise cancer risk data are very limited. If colorectal cancer does develop, however, the prognosis is recognized to be poor with reduced survival^[4]. Several studies, again from tertiary care centers, have suggested that patients with Crohn's disease have an increased risk of colorectal cancer^[5,6] and an excess overall mortality attributed to digestive tract tumors, including small bowel carcinoma^[7]. The latter occur at a younger age, usually in males compared to those with small bowel carcinoma unrelated to Crohn's disease^[8].

Weedon *et al*^[5] reported colorectal cancer in 8 of 449 patients with Crohn's disease, or about 1.2% (i.e., an estimated 20 times greater risk than a control population). Similarly, Gyde *et al*^[6] described an approximately 4-fold increased risk in patients with Crohn's disease. More recent cohort and population-based studies from Canada, where reporting of malignant disease is legally mandated^[7,9], are also consistent with an increased intestinal cancer risk in Crohn's disease. In Europe, north-south differences in intestinal and extra-intestinal cancers have also been recently noted^[10]. Interestingly, in Asia, with Crohn's disease now dramatically increasing, there is a high rate of colorectal cancer, particularly in the lower rectum and anal area^[11]. A recent and extensive meta-analysis has also recently confirmed the increased colorectal and small bowel cancer risk in Crohn's disease^[12]. Moreover, other malignancies have been reported in Crohn's disease, including myeloid and lymphoid malignancies^[13], possibly related, in part, to wider use of immunosuppressants or biological agents (e.g., infliximab)^[14,15]. Finally, carcinoid tumors may be increased

in Crohn's disease^[16], and this has recently been estimated as a 15-fold risk^[17].

RISK FACTORS

In a cohort-based study of Crohn's disease followed over more than 2 decades, 1% had intestinal cancers detected^[13]. The clinical features of the intestinal cancers included: a long history of Crohn's disease, often (but not exclusively) over 20 years predating cancer development; a relatively young age of intestinal cancer diagnosis in Crohn's disease; and, the appearance of other histopathological types, including mucinous adenocarcinoma. Most cancers occur in the distal colorectum, often in the presence of extensive inflammatory disease. Cancers were also detected in bypassed or excluded segments of intestine, including rectal "stump" cancer, a potentially important and independent risk factor for later cancer development following colonic resection. The prognosis has also been disconcerting as disease is often detected late and mortality has been significant^[8]. Even though epithelial dysplasia (thought to be a neoplastic intestinal marker for later or concomitant invasive cancer) has been defined in both small and large intestine supporting the concept of a dysplasia-carcinoma sequence in Crohn's disease, most cases of intestinal cancer, even in large tertiary care centers, are discovered incidentally at the time of surgical resection for treatment of the Crohn's disease.

FUTURE RESEARCH

To date, specific recommendations for screening and surveillance colonoscopy, even in chronic and extensive Crohn's colitis, have been supported by only very limited data in older patients^[18]. Indeed, it can be anticipated that the focal nature of dysplasia (as occurs even in extensive ulcerative colitis) may make detection of dysplasia even more difficult in Crohn's disease, a disorder generally characterized by patchy or segmental inflammatory change. As a result, establishing a productive screening program for epithelial dysplasia or focal cancers in Crohn's disease can be expected to prove difficult, even with dye staining or the intriguing potential of newly evolving technologies, such as confocal microendoscopy. Even in extensive colitis, a recent report found that colonoscopy surveillance may not improve survival, but only detect cancers at an earlier stage^[19]. Other tools that might predict later cancer development in Crohn's disease, employing molecular or genetically-based markers^[20], are still desperately needed and should be aggressively pursued.

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REVIEW

Role of chemotherapy and novel biological agents in the treatment of elderly patients with colorectal cancer

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Abstract

Patients older than 65 years are the fastest growing segment of the cancer population. It is estimated that within 20 years over 75% of cases and 85% of deaths from colorectal cancer (CRC) will be in this setting. Concerns about cancer treatment in the elderly relate to comorbidities, which increase proportionally with age, physiological changes associated with aging which may influence drug metabolism and toxicity, and diminishing life expectancy, which particularly impacts decisions surrounding the benefits of adjuvant therapies. Over the last 10 years, significant improvements in the treatment of advanced CRC with combination therapy have been made. The randomized trials which have defined these improvements did not exclude elderly patients. However, the median age of patients in these trials has generally been approximately 60 years. Thus, it appears that some degree of selection is involved with younger and presumably fitter patients being the subjects in most of the pivotal trials. The availability of new molecularly targeted agents and newly improved existing agents has expanded the range of treatment options available. This variety gives greater flexibility in dealing with different subsets of patients, such as the elderly. However, some fit elderly patients seem to tolerate combination therapy reasonably well, while studies on unfit elderly subjects are needed.

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Key words: Bevacizumab; Chemotherapy; Cetuximab; Colorectal cancer; Elderly patients

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INTRODUCTION

Advancing age is often associated with an increase in cancer diagnosis. Malignancies represent the second cause of death in the elderly population in the Western countries, and this age group represents more than half of all diagnosed cancers. Due to a continuous increase in life expectancy, we may expect a higher rate of older patients with malignant disease in the future, and a growth in health expenses. However, to date few data are available in the literature about the treatment of this group of patients. Elderly patients have been under-represented in or excluded from clinical studies, mainly because older age is chosen to be an exclusion criterion^[1]. Very often, these patients are not treated because many believe the cancer growth potential to be lower in older subjects than in younger ones. Thus, many elderly cancer patients receive general supportive care. If this choice is a valid option for that group of patients defined as “frail”, this is not justifiable for all elderly patients. Often, oncologists fear heavy toxicities or suffer the patients and their relatives prejudice toward collateral effects resigning chemotherapy. However, in the Royal Marsden Hospital, no statistically significant difference in the overall or severe toxicity between the population aged 70 or older or the younger cohort was observed during adjuvant treatment for colorectal cancer (CRC) with a 5-fluorouracil (5-FU)-based chemotherapy^[2]. The only exception was stomatitis, which was more frequent in the older age group (19% *vs* 11%, *P* = 0.01). Regardless, when one plans a chemotherapy treatment in an older patient it is necessary to take into consideration the incidence and severity of myelosuppression, mucositis, nausea and vomiting, cardiomyopathy and peripheral neuropathy can increase above 70 years of age. On this basis, it is necessary to individualize a strategy to better tailor the treatment plan at the individual level. The assessment of the functional status by means of the widely used Karnofsky or Eastern Cooperative Oncology Group (ECOG)

Table 1 Comprehensive geriatric assessment

Measure	Description
Function	Activities of daily living (ADL) Instrumental activities of daily living (IADL) Performance status (PS)
Health	Number of co-morbid conditions
Socio-economic status	Income, education, living conditions, caregiver
Geriatric syndromes	Dementia (Mini-mental state examination, MMSE) Delirium Depression (Geriatric depression scale, GDS) Incontinence, failure to thrive, neglect and abuse
Pharmacy	Polypharmacy (other medications being taken)
Nutrition	Mini nutritional assessment

does not seem as effective in older patients as in the adult population, because comorbidities in the elderly may interfere with the measurement of the performance status (PS)^[3]. Several instruments have been proposed to monitor comorbidities, although none has been validated or widely accepted by the oncologic community^[4]. A Comprehensive Geriatric Assessment (CGA) scale was thus developed and validated by the Italian Group for Geriatric Oncology (GIOGer) (Table 1)^[5]. The functional, emotional and cognitive status, comorbidities number, and the numbers of those with depression and geriatric syndromes may help to better define populations that may or may not benefit from various therapeutic approaches (Table 2). Another problem is the definition of “elderly” patient. There is a widely variable perception of the age at which a patient is considered elderly, and this is based on chronological rather than physiological age. In studies of the treatment of acute myeloid leukaemia, patients over 60 were considered elderly while patients with solid tumors had to be over 70^[6]. These differences make data comparison among clinical studies more difficult. Besides these factors, changes in the elderly also occur in terms of the functions of several organs. Noteworthy are alterations in kidney and liver functions as well as the apparent bone marrow reserve. In addition, elderly patients very often have additional medication, which may significantly influence the *p450* cytochrome function. For this and other reasons clinicians are unwilling to treat an elderly patient. This paper will review the current therapeutic armamentarium suitable for CRC patients and its applicability to elderly subjects both in the adjuvant setting and in advanced disease.

ADJUVANT CHEMOTHERAPY

Patients with newly diagnosed CRC have a median age of 70 years. Local recurrence or distant metastases are frequent within the first two years. The mean life expectancy of a 65-year-old man is approximately 13 years and for a 65-year-old woman the mean life expectancy is estimated to be nearly 19 years. Thus, an effective reduction in the occurrence of a disease relapse due to an adjuvant chemotherapy may be of major importance for these patients, as their life expectancy exceeds the time in which the appearance of metastatic disease would compromise their sur-

Table 2 Classification of patients into 3 treatment categories based on CGA

Group	Description	Treatment
1	Healthy, good PS	Standard cancer treatment
2	Partially dependent, ≤ 2 comorbidities Life expectancy shortened by cancer	Standard cancer treatment if can tolerate treatment Palliation if cannot tolerate treatment
	Life expectancy not shortened by cancer	Palliation
3	Frail patients who are totally dependent with ≥ 3 comorbidities or 1 geriatric syndrome	Palliation

vival. On the other hand, a pooled analysis of individual patient data from seven phase III randomised trials (involving 3351 patients) in which the effects of postoperative 5-FU plus leucovorin (LV) or levamisole were compared with the effects of surgery alone in patients with stage II or III colon cancer has demonstrated a benefit in terms of overall survival (OS) in each age group^[7]. The patients were grouped into four age categories of equal size, and analyses were repeated with 10-year age ranges (≤ 50, 51 to 60, 61 to 70, and > 70 years). OS and the time to tumor recurrence were significantly longer in patients treated with 5-FU-based therapy than in patients who did not receive adjuvant treatment ($P < 0.001$). No significant interaction was observed between age and treatment effect for OS or freedom from tumor recurrence, regardless of how age was included in the analysis. The survival curves for the patients who were older than 70 years of age converged slightly after five years, probably because of deaths from other causes. Analyzing the toxicities according to age for the two treatment regimens, the authors found age was not significantly related to the rate of grade 3 or higher nausea or vomiting, stomatitis or diarrhea among patients treated with either 5-FU plus LV or 5-FU plus levamisole. Although increased age was associated with higher rates of severe leukopenia in patients treated with 5-FU plus levamisole ($P \leq 0.001$), this relationship was of borderline significance in patients who received 5-FU plus LV ($P = 0.05$). However, this analysis denotes some critical aspects. The principal limitation of this study concerns its potential applicability to the general population of elderly patients. As a result of exclusion criteria and screening, elderly patients who enter clinical trials are a select group, with good PS, easy access to transportation and limited numbers of comorbidities. How co-existing conditions, malnutrition and poor social support might affect the efficacy and tolerability of 5-FU-based chemotherapy is unknown. It will be up to further studies to explain the decision to treat an elderly patient who has several other problems involving physician, patient and family. Moreover, only 23 of the 3351 patients (0.7%) in the trials were over the age of 80 years. Caution is therefore advised in extrapolating these findings to octogenarians.

Capecitabine is being investigated for the treatment of elderly patients with CRC. The X-ACT trial, comparing

oral capecitabine monotherapy (1250 mg/m² twice daily for 2 wk on/1 wk off) with the Mayo Clinic regimen (bolus 5-FU 425 mg/m² days with LV 20 mg/m² days 1-5 every 4 wk) in the adjuvant setting among 1987 patients with Dukes' C colon cancer, reported significantly superior relapse-free survival with capecitabine ($P = 0.041$), and fewer adverse events than with 5-FU plus LV ($P = 0.001$)^[8]. As a result, capecitabine monotherapy is now approved for adjuvant therapy of Dukes' C colon cancer. Diaz-Rubio *et al* provided a retrospective safety analysis on a subpopulation of patients ≥ 70 years of age (capecitabine: $n = 186$; 5-FU/LV: $n = 205$) from the X-ACT trial database^[9]. With respect to all-grade non-hematologic adverse events, elderly patients treated with single-agent capecitabine had significantly less diarrhea (52% *vs* 68%, $P = 0.002$), stomatitis (23% *vs* 67%, $P < 0.001$), and nausea (33% *vs* 47%, $P = 0.005$) than patients treated with bolus 5-FU/LV. Only all-grades hand-foot syndrome (HFS) was seen significantly more frequently with capecitabine (63% *vs* 8%, $P < 0.0001$). With respect to grade 3 or 4 hematologic adverse events, elderly patients had significantly less neutropenia with capecitabine than 5-FU/LV (4% *vs* 31%, $P < 0.00001$). Grade 3 or 4 hyperbilirubinemia was significantly greater with capecitabine than 5-FU/LV, when measured by NCI Common Terminology Criteria for Adverse Events. Although these results are promising, additional efficacy, quality of life (QoL), and cost data, particularly from the X-ACT trial, are needed to assess the usefulness of capecitabine for the treatment of elderly patients with CRC in the adjuvant setting.

A recent retrospective, age-based ($<$ or ≥ 70 years), pooled analysis including 3742 CRC patients (614 age ≥ 70) was conducted extrapolating data in the Sanofi-Aventis database from four clinical trials testing the combination of oxaliplatin plus 5-FU/LV administered bimonthly (FOLFOX4) in the adjuvant, first-, and second-line settings^[10]. End points included grade ≥ 3 adverse events, response rate (RR) (in advanced disease), progression or relapse-free survival, dose-intensity, and OS in the studies with mature survival data. The advantages of FOLFOX4 have been demonstrated in stage III patients^[11]. The four trials formed the basis for the US Food and Drug Administration approval of FOLFOX4 in the treatment of metastatic CRC (first- and second-line settings) in stage III patients (after complete surgical resection). There was no difference in efficacy derived between younger and older patients enrolled into these trials with respect to RR, relapse/progression-free survival or OS. The analysis showed similar toxicity patterns in the two age groups. Increased rates of neutropenia (43% *vs* 49%; $P = 0.04$) and thrombocytopenia (2% *vs* 5%; $P = 0.04$) were observed in the older patients. However, efficacy outcomes were not different between the two age groups. In addition, drug delivery doses did not differ significantly by patient age and there was no difference in the incidence of treatment-associated deaths or neuropathy as a consequence of age. However, older patients who enrolled in these trials clearly are a select group, suggesting that generalizations derived from this study must be applied cautiously to individual older patients.

CHEMOTHERAPY FOR ADVANCED DISEASE

5-FU

Treatment of patients with metastatic disease is palliative. As for any other age group of patients, concern may be raised whether an elderly patient might benefit most from general supportive care rather than from toxic treatments. If one considers that patients with a new diagnosis of CRC have a median age of 70 years, the first endpoint remains symptoms palliation or clinical benefit, and not the objective response (OR) or OS time. Renal elimination of 5-FU after its catabolism in the liver and mucosa is limited and estimated to account for no more than 10% of excreted drug^[12].

Therefore, 5-FU dose reduction in patients with renal dysfunction (possible in the older population) is usually not considered necessary. On the other hand, a large amount of 5-FU has to be metabolized by extrahepatic tissue. On this basis, a mild decrease in renal or hepatic function related to age is not a sufficient reason to reduce a 5-FU dose. A study examined the potential influence of gender and age on 5-FU-clearance^[13]. Both factors are considered to have potential roles in the pharmacokinetic variability of drugs. There was no evidence that age modified 5-FU-clearance when it was adjusted for sex and dose. Female sex turned out to be a major determinant for increased toxicity, while age was not. These data quite justify the use of this drug in elderly patients. A large number of clinical trials confirm these assumptions. An Italian Group treated patients with a median age of 75 (range 70 to 85 years) with best supportive care or a weekly 5-FU bolus and LV regimen^[14]. Interestingly, the median survival of the patients receiving chemotherapy was prolonged by 2 mo, indicating a potential benefit of chemotherapy in the elderly, and so confirming data from studies in the younger population. Adverse events were reversible and of limited impact. The study did not show any grade 4 toxicity, while grade 3 toxicity was verifiable in only 16.4% of cases. Similar encouraging results were reported in trials employing 5-FU continuous infusion (c.i.), which decreases the hematological toxicity. Two Italian phase II experiences evaluated the efficacy and safety of the "de Gramont" schedule in patients aged 70 years or older^[15,16]. Both these trials reported ORs in 20% of patients and median survivals of about 12 mo, but demonstrated the feasibility of chemotherapy in elderly patients without quality of life worsening and with improvement of symptoms. In an attempt to anticipate the risks and benefits of chemotherapy, the authors applied the geriatric assessment scales (ADL and IADL) to patients. Unfortunately, neither of these scales was useful to these aims. However, these studies were carried out on a very small sample and there was a high risk of false-negative results. Regarding side effects, gastrointestinal and hematological toxicities were common, but rarely severe. A recent pooled retrospective analysis of data regarding 3825 patients (629 aged 70 years or older) included in 22 European phase II and III trials analyzed the role of 5-FU in the treatment of advanced disease^[17]. The majority

of elderly patients were aged 70 to 74 years. They were generally treated with bolus 5-FU and its modulation by LV, methotrexate or interferon and less with 5-FU c.i. The results indicated 5-FU-based chemotherapy had the same activity in elderly patients compared with younger subjects, in terms of ORs (23.9% *vs* 21.1%, respectively; $P = 0.14$), progression-free survival (PFS) (5.5 *vs* 5.3 mo, respectively; $P = 0.01$) and OS (10.8 *vs* 11.3 mo, respectively; $P = 0.31$). The 5-FU c.i. allowed a small improvement in these results. Although no significant differences were observed between age and treatment efficacy, the number of subjects over the age of 75 years was only 3.8%. Moreover, elderly patients who entered clinical trials were a select subgroup, with limited comorbidities, and probably not representative of the general older population. Totally absent were the toxicities data in this analysis. Reports on the efficacy and toxicity of 5-FU-based-chemotherapy for that group of patients defined as “frail” are lacking in literature.

Raltitrexed

Raltitrexed is a nonfluoropyrimidine thymidilate synthase inhibitor that has shown efficacy and tolerability in the treatment of CRC. A randomized trial for metastatic disease demonstrated equal efficacy of raltitrexed compared with a conventional 5-FU-bolus regimen in terms of OR and OS^[18]. However, leukopenia and mucositis were more frequent in the 5-FU-based arm than in the experimental arm. The once-every-3-wk dosing, tolerability profile and ease of administration advocated for further investigation in elderly population. However, 50% of raltitrexed is excreted by the kidney. In the case of renal dysfunction and creatinine clearance decreasing, the dose of drug has to be adapted. The higher rate of therapy-associated deaths due to a failure to adapt the raltitrexed dose in patients with renal dysfunction accounts for the premature closing of the Pan-European Trial on Adjuvant Colon Cancer (PETACC 1)^[19]. The use of raltitrexed may be justified in subjects with 5-FU-associated cardiotoxicity^[20]. As older patients are more likely to have a cardiovascular disease, and patients with pre-existing cardiovascular disease are more likely to experience 5-FU-associated cardiotoxicity, the use of raltitrexed in this age group may be of potential benefit. Two studies have evaluated the efficacy, safety and toxicity of raltitrexed in patients aged 70 years and older^[21,22]. Treatment with raltitrexed resulted in clinical improvement of tumor-associated symptoms in 38% of cases and was associated with an acceptable toxicity profile. In particular, a risk group for nausea-vomiting and diarrhea was females between 70 to 75 years old, and a risk group for liver toxicity was males aged > 75 years. On the basis on these results the authors suggested raltitrexed was a suitable option in elderly patients.

UFT

UFT combines the dihydropyrimidine dehydrogenase (DPD) inhibitor uracil with the 5-FU prodrug tegafur in a 4:1 molar ratio. Uracil competes with 5-FU for DPD and inhibits the degradation of the 5-FU generated by tegafur^[23]. Compared with 5-FU alone, administration of UFT results in higher concentrations of 5-FU in tumors^[24].

Two large, multinational phase III trials compared UFT plus LV *versus* the Mayo regimen of 5-FU and LV in patients with previously untreated advanced CRC. A regimen of UFT (300 mg/m² per day) plus oral LV (75 or 90 mg per day) for 28 d every 5 wk was compared with 5-FU (425 mg/m² per day) plus LV (20 mg/m² per day) intravenously for 5 d every 4 wk or 5 wk^[25,26]. The larger study with 816 patients reported similar OR rates (12% for UFT plus LV *vs* 15% for 5-FU plus LV) and no statistically significant difference in survival times. In the second study, which included 380 patients, the two regimens demonstrated similar times to disease progression, median survival times and RRs. However, in both studies, the UFT plus LV regimen showed significantly lower toxicity, with a lower incidence of grade 3 mucositis, myelosuppression, febrile neutropenia, and infections, and no notable hand-foot syndrome. These advantages make this form of oral therapy suitable for elderly patients. So, two Spanish groups reported good tolerability and efficacy for the use of UFT in elderly patients with metastatic CRC^[27,28]. A recent ECOG trial evaluated the RR and toxicity profiles of elderly subjects, defined as those ≥ 75 years of age, treated with UFT and LV^[29]. Treatment was administered as UFT (100 mg/m²) plus LV (30 mg) every 8 h for 28 d with 7 d of rest. Fifty-eight patients were enrolled with a median age of 81 (range, 75-89). Fifty-seven patients were evaluable for toxicity with grade 3-4 as follows: Gastrointestinal 20 (34%), neutropenia 4 (7%), no hand-foot syndrome. There was only one case of grade 4 diarrhea reported. In six cases (10%), a dose reduction for gastrointestinal toxicity was required, while there were 2 fatalities with gastrointestinal bleeds. The RR was 19%, median time to progression (TTP) was 19 wk, and OS was 11.8 mo. Thus, UFT + LV was well tolerated in this study with an incidence of grade 3-4 toxicity similar to phase III reports in younger patients. Activity was comparable to intravenous 5-FU/LV. This oral fluoropyrimidine is well tolerated and very active in elderly patients.

Capecitabine

Capecitabine, an oral formulation of 5-FU, was developed as an alternative to intravenous 5-FU. Compared with the parenteral compound, capecitabine provides greater tumor selectivity while minimizing systemic exposure. The drug is well absorbed via the gastrointestinal tract and is catabolized to active drug by a series of three enzymes. Over 70% of the metabolites are excreted by the kidney. This makes one cautious when it is necessary to treat an elderly subject. A moderate restriction in liver function does not appear to alter the pharmacokinetic of this drug in a clinically relevant fashion^[30]. A large, randomised, open-label phase II trial conducted in Europe, North America and Australia evaluated three schedules of capecitabine (continuous, intermittent and intermittent with oral LV) in patients with metastatic CRC^[31]. The addition of LV seemed to increase the incidence of side-effects without any benefit to RR or survival times. The RR for the three schedules ranged from 21% to 24%; the median time to disease progression ranged from 127 d to 230 d, with the longest TTP being seen in the capecitabine intermittent arm (with-

out LV). This schedule, which consists of twice-daily dosing for 14 d followed by 7 days' rest, was further evaluated in two phase III trials. Each of these large trials included more than 600 patients. In the trial conducted in 61 centers in the USA, Brazil, Canada, and Mexico, a total of 605 patients were randomized to receive either 2500 mg/m² per day capecitabine in divided daily doses for 14 d followed by 7 days' rest or the Mayo regimen described above^[32,33]. Capecitabine was more active than 5-FU in the induction of a tumor response, and the two groups showed similar times to tumor response and response durations. TTP and OS times were comparable between the two regimens, but the toxicity of capecitabine was less than that of 5-FU, with a substantially lower incidence of diarrhea, stomatitis, nausea and alopecia. However, capecitabine was associated with a higher incidence of palmar-plantar erythrodysesthesia. While capecitabine was shown to be tolerated by fit elderly patients, until a short time ago information on dosing and scheduling for older patients with impaired organ function was not available. Recently, a multicentre phase I / II trial of capecitabine (2000 mg/m² per day for 14 d every 3 wk) was conducted in 214 patients aged ≥ 65 years and/or with an ECOG PS ≥ 1 ^[34]. In the 192 patients evaluable for toxicity, there were no grade 3-4 hematological toxicities. Grade 3-4 toxicity occurred in 22% of patients during the first 3 cycles (8.9% HFS, 6.3% diarrhea, 2.6% lethargy, 2.6% dehydration, 1% abdominal pain, 0.5% stomatitis). Dose reductions were required in 14% and dose delays in 21% for medical reasons. In the 151 evaluable for activity, a response was seen in 13%, median PFS was 5.1 mo, and median OS was 16.3 mo. The authors demonstrated lower dose capecitabine was tolerable and active in less fit patients. This study provides valuable information on possible outcomes in these under-studied patients for whom combination chemotherapy may not be preferred. Oral therapies avoided central access devices, with their attendant costs, inconvenience to patients and potential for costly and morbid complications. These factors, along with patient preference for an oral regimen, have contributed to the development of oral 5-FU preparations.

Irinotecan

Irinotecan (CPT-11) is a semisynthetic derivative of the natural alkaloid camptothecin, and belongs to a new class of antineoplastic agents called topoisomerase I interactive compounds. Since its introduction into the clinic, CPT-11 has undergone a comprehensive evaluation as a single agent and in combination chemotherapy in first-line as well as in second-line therapy of CRC. In two studies using either infusional or 5-FU bolus regimens, CPT-11 was able to improve the objective RR as well as the median survival of patients receiving 5-FU plus LV and CPT-11 combination therapy^[35,36]. However, the inclusion criteria of both trials prevented patients over 75 years of age from being treated within the protocol. CPT-11 used as a single agent is associated with equal toxicity in younger and fit older patients (above 65 years of age)^[37]. Pharmacokinetic studies have demonstrated equivalent drug pharmacological parameters in patients below or above 75 years of age^[38]. A retrospective analysis compared toxic-

ity and survival according to age during a CPT-11-based treatment of 339 patients with fluoropyrimidine-resistant advanced CRC. All patients commenced CPT-11 at 350 mg/m² once every 3 wk and of the 339 patients, 72 (21%) were aged ≥ 70 ^[39]. There were no differences in the proportions of patients developing toxicities by age (< 70 vs ≥ 70 : 37.8% vs 45.8%; $P = 0.218$). Patients aged ≥ 70 had similar ORs (11.1% vs 9%; $P = 0.585$) and survival (median 9.4 vs 9 mo; $P = 0.74$) compared with younger patients. These data suggest elderly patients derive the same benefit without experiencing more toxicity with second-line CPT-11 treatment for advanced CRC, and do not support the recommendations to give reduced starting doses to elderly patients. Although in a phase II study older patients were twice as likely (38.6% vs 18.8%; $P < 0.008$) to develop grade 3-4 diarrhea compared with younger patients^[40], and although clinicians often reduce the dose of CPT-11 from 350 mg/m² to 300 mg/m² when administered in a three-weekly schedule or from 125 mg/m² to 100 mg/m² in the weekly schedule, this is rather a precaution than an evidence-based indication. Moreover, a recent small retrospective study has demonstrated irinotecan (80 mg/m²) is active and tolerable when administered once a wk for 2 wk, followed by a wk rest in pretreated CRC patients aged 70 years or more^[41]. The most frequently observed severe toxicities were diarrhea (grade 3, 13%) and neutropenia (grade 3, 30.4%; grade 4, 8.6%). Only one case of neutropenic fever occurred. Other hematological and non-hematological toxicities were mild and manageable. Objective partial responses (PR) were observed in 13% of cases and an additional 43% of patients reported a stable disease (SD). Just the lack of exhaustive data in literature has justified some recent trials evaluating the efficacy and safety of CPT-11 in combination regimens in elderly patients. In an Italian phase I / II trial accepting pretreated older patients, irinotecan in combination with oxaliplatin (OXIRI) were evaluated through a weekly schedule^[42]. Twenty-one patients were enrolled at the second dose level with the maximum tolerated doses of 40 mg/m² for oxaliplatin and 60 mg/m² for CPT-11. The obtained results demonstrated the feasibility of chemotherapy with a good toxicity profile and acceptable efficacy (RR 28%). A Spanish phase II study evaluated the combination of CPT-11 and 5-FU 48 h c.i. as a first-line chemotherapy for patients older than 72 years^[43]. Inclusion criteria such as Karnofsky > 70 , adequate hepatic and renal function, normal blood cell counts and absence of geriatric syndromes were required. Although treatment delay was observed in 39.7% of cases, particularly for hematological toxicity, and dose reduction was required in 19% of subjects both for hematological and non-hematological toxicity, grade 3-4 toxicities appeared in about 20% of cases. Peripheral venous thrombosis was reported in 4 cases, central venous catheter thrombosis in one case and pulmonary embolism in yet another one. There were 2 toxic deaths, one due to grade 4 diarrhea and acute renal failure and the other due to a pulmonary embolism reported as unrelated to the treatment. Forty-four patients were assessable for efficacy with a RR of 31.8%. Thirty consecutive, previously untreated patients (76 years median age) with metastatic CRC, were

enrolled in another phase II trial evaluating the FOLFIRI regimen^[44]. Although this combination appeared manageable (grade 3-4 neutropenia 20%; grade 3 thrombocytopenia 3.3%; grade 3 asthenia 10%; grade 3-4 diarrhea 17%), one treatment-related death due to neutropenic sepsis was registered. Overall, RR was 36.6% and the median TTP was 7 mo. After a median follow-up period of 17 mo, the median OS was 14.5 mo. A combined analysis of 2691 patients included in randomized trials has been recently presented to compare the efficacy and toxicity in older (≥ 70 years) and younger (< 70 years) subjects receiving first-line 5-FU/FA with or without irinotecan^[45]. There was no imbalance regarding risk factors (ECOG PS, WBC, number of tumor sites, alkaline phosphatase and LDH) between elderly and younger patients. Older and younger patients had significantly improved RRs and PFS with combination therapy than with 5-FU/FA. Younger patients had significantly longer OS with irinotecan and 5-FU/FA ($P = 0.0003$), while older patients had a trend to longer OS with this combination therapy ($P = 0.15$). The combination was associated with more grade ≥ 3 toxicity in the general population, but there were no significant differences regarding toxicity between older and younger patients. Although this analysis has considered patients aged over 70 years who were selected for inclusion in phase III trials, it has demonstrated elderly patients derive similar benefits from irinotecan-containing chemotherapy, and with similar risks of toxicity, compared with younger patients. Two studies reported preliminary data on the efficacy and tolerability of CPT-11 in combination second-line regimens in patients aged ≥ 66 ^[46,47]. The first of these was conducted adopting a weekly schedule of CPT-11 and bolus 5-FU in 10 patients who had relapsed or had progressive disease after oxaliplatin-5-FU/LV combination. Three of the 10 patients showed a PR. The median TTP and median survival time were 4.5 and 12 mo, respectively. However, the toxicity profile was burdened with these percentages of grade 2-3 adverse events: Neutropenia 50%, thrombocytopenia 22%, anaemia 33%, diarrhea 33%, nausea 44% and fatigue 39%. The second trial evaluated the safety and efficacy of CPT-11 plus capecitabine. The 26 enrolled patients received first-line chemotherapy with FOLFOX4 in 16 cases, FOLFIRI in 4, and 5-FU/LV/methotrexate in 6 cases. Eight of 24 evaluable patients (33%) showed a response to treatment, median TTP was 5.5 mo, and OS was 11.5 mo. The most common grade 3 side effects were diarrhea (40%), nausea and vomiting (20%), and hand-foot syndrome (10%). Grade 3-4 neutropenia was seen in 40% of patients. No treatment-related death was reported. Nevertheless, more data on the use of CPT-11 in elderly patients would be reassuring.

Oxaliplatin

Oxaliplatin is a novel platinum derivative and the first platinum compound to demonstrate significant efficacy in the treatment of advanced CRC. *In vitro* and *in vivo* preclinical studies on CRC have shown oxaliplatin is active against colorectal cell lines and is synergistic with 5-FU^[48]. In one randomized trial, accepting patients below the age of 75 years, the role of oxaliplatin in combination with 2-d

administration of high-dose LV plus 5-FU bolus and low-dose infusional 5-FU in the first-line therapy of advanced CRC was evaluated in 420 patients^[49]. The objective RRs in elderly and younger patients treated with infusional 5-FU/LV (22.2% *vs* 21.4%, respectively) were not different from those treated with infusional 5-FU/LV plus oxaliplatin (50% *vs* 50%; > 65 years, $n = 160$) as compared to younger patients, respectively. In general, compared with younger patients, this group of elderly patients did not experience increased toxicity except for grade 3-4 diarrhea (18% *vs* 8%, $P = 0.34$). The combination regimens employing oxaliplatin plus infusional 5-FU/LV have less hematological toxicity, in particular for FOLFOX2 and FOLFOX6. Thus, clinicians have had a preference for oxaliplatin compared with CPT-11 when they have considered possible the evaluation of a polychemotherapy for elderly patients in several recent phase II studies. An Italian study assessed the tolerability and efficacy of FOLFOX2 in the treatment of pretreated and metastatic elderly patients in comparison to a series of patients < 65 years^[50]. The preliminary data suggested FOLFOX2 had comparable activity between the two groups (RR 30%) and this schedule was well tolerated in the elderly group. The main toxicities, albeit of short duration, were neutropenia, mucositis, diarrhea, and neurotoxicity. A tailored regimen including capecitabine and oxaliplatin (XELOX) for treating elderly patients with metastatic CRC was planned on September 2001^[51]. Thirty-five patients aged 70-81 years were treated with an alternated dose escalation for both drugs over the first 3 cycles for each patient in the absence of WHO grade ≥ 2 toxicity on previous cycle. Starting doses were 85 mg/m² for oxaliplatin on d 1, and 2000 mg/m² for capecitabine, which was taken orally, twice a day, from d 2 to d 15. Dose escalation was performed in 51% of patients for oxaliplatin, and in 11% of cases for capecitabine. No grade 4 and 10 (29%) cases of grade 3 toxicity of any type were reported. Abdominal symptoms (pain, nausea or vomiting) affected 66% of patients, but they were of grade 3 in only 2 patients. Grade 3 diarrhea occurred in 9% of patients. The overall RR was 40%, while PFS and OS time were 6.9 and 14.1 mo, respectively. The authors reported compliance was fairly good considering only one patient went off for refusal in this study. Another three studies have investigated the XELOX regimen as first-line treatment for elderly patients with CRC^[52-54]. Even if there was one treatment-related death for diarrhea in two of these trials, the authors emphasized the tolerability of this regimen for elderly patients. Thus, in the Feliu *et al* experience, reporting a median relative dose intensity of 92% for oxaliplatin and 98% for capecitabine with a RR of 36% and a TTP of 6.9 mo, the more frequent grade 3-4 toxicities per patient were: Diarrhea 22%, asthenia and vomiting 14%, nausea 10%, and anorexia 8%^[53]. In the Comandone *et al* trial employing the same combination, 27 patients, 8 of whom were pretreated with chemotherapy, entered the study^[54]. Following the RECIST criteria the authors observed a RR of 19.2%, while the median TTP and OS were 6.1 and 14.2 mo, respectively. The grade 1-2 toxicities were: Peripheral neuropathy 40%, nausea-vomiting 18%, neutropenia 26%, and asthenia 35%. In

one only case was the treatment interrupted for grade 3 neuropathy after one course of therapy. We have tested the oxaliplatin plus oral UFT/LV combination as a first-line therapy in patients with advanced or metastatic CRC aged 70 or older^[55]. Forty-seven patients aged ≥ 70 were treated with oxaliplatin 65 mg/m² as an intravenous 3-h infusion on d 1 and 8 plus UFT 300 mg/m² and LV 90 mg in three divided doses given orally on d 1-14 for each 3-wk cycle. Patients were followed by a geriatric and a QoL assessment with specific scales and EORTC-QLQ-C30 questionnaire. All patients were assessable for toxicity and 45 for response to treatment. The overall RR was 51%, and the median duration of response was 8 mo (range, 3-19+ mo). After a minimum follow-up of 17 mo, the median TTP and the median OS were 8.0 and 14.1 mo, respectively. Regimen safety was manageable. Most adverse events were mild to moderate, and this did not result in QoL impairment. The most common grade 3-4 treatment-related adverse events were diarrhea (17%), neutro- and thrombocytopenia (2%), laryngeal spasm (2%), and peripheral neuropathy (12.7%). No treatment-related death occurred. In addition, early phase II data for modified FOLFOX4 are available^[56,57]. Massaccesi *et al* examined 78 patients aged ≥ 70 years and ECOG performance status ≤ 2 . Their schedule was: Oxaliplatin 45 mg/m² + 5-FU bolus 400 mg/m² + 5-FU infusional 600 mg/m² + LV 200 mg/m², d 1 and 2, every 2 wk. Responses and geriatric scales ADL and IADL were assessed every 6 cycles. The overall RR was 50.5%, the median duration of response was 9 mo and second-line chemotherapy was delivered to 47% of patients. With a median follow-up time of 12.5 mo, the median TTP and OS were 8.1 and 20.1 mo, respectively. The main grade 3-4 toxicities were: Neutropenia 32%, diarrhea 10%, mucositis 4% and fatigue 4%. Grade 2 and 3 sensory neuropathy occurred in 17% and 6% of cases, respectively. Bi-fractionated FOLFOX4 was highly active and it demonstrated reduced neurotoxicity^[56]. In the Kim *et al* experience to minimize toxicity and improve compliance of chemotherapy in elderly patients, reduced dose intensity (mini-) FOLFOX4 regimen was used as a first-line palliative chemotherapy. The schedule was: Oxaliplatin 65 mg/m² on d 1 + 5-FU bolus 300 mg/m², 5-FU infusional 450 mg/m², and LV 150 mg/m² on d 1 and 2, every 2 wk. Twenty-seven patients older than 70 years of age were enrolled. The overall RR was 31.8%, median PFS and OS were 7.1 and 13.5 mo, respectively. The main side effect was grade 1-2 anemia and neutropenia, observed in 24.3% and 13.5% of patients, respectively. There were no grade 4 toxicities and only one patient suffered from grade 3 neuropathy and vomiting. The authors recommended mini-FOLFOX4 in elderly patients with advanced CRC being well tolerated with acceptable toxicity without compromising objective RR or survival^[57].

NOVEL BIOLOGICAL AGENTS

Cetuximab

Cetuximab is a monoclonal antibody against the epidermal growth factor receptor (EGFR), which is expressed in many patients with CRC. Addition of cetuximab to

chemotherapy improved outcomes both in previously treated and in untreated patients^[58-60]. Only one study has evaluated the activity and safety of cetuximab as a single agent in the first-line treatment of elderly patients^[61]. Forty-one patients ≥ 70 years old with confirmed metastatic CRC, Karnofsky PS ≥ 80 , and adequate renal, hepatic and bone marrow function were included. Cetuximab (400 mg/m² as initial dose and 250 mg/m² weekly thereafter) was administered until progressive disease, unacceptable toxicity or consent withdrawal. Only two patients required dose reduction of cetuximab due to toxicity, and there was a dose delay of one wk in 12 cases (29%), achieving a median relative dose intensity of 80%. The main toxicities were those expected for cetuximab: Acne-like rash grades 1-2 (54%), grade 3 (10%), nail toxicity grades 1-2 (7%) and infusion related toxicity grades 1-2 (5%). Thirty-nine patients were evaluable for efficacy: One showed a complete response (CR), 5 showed a PR, 15 showed SD and 18 showed progressive disease (PD), resulting in an overall RR of 15.4% and tumor growth control of 54%. Cetuximab monotherapy is feasible in elderly patients as a first-line treatment for metastatic CRC with a favourable safety profile. Response and disease control rates remain in the range observed in pretreated patients. Further research with cetuximab in combination therapies is warranted in this population, as it could improve the efficacy of chemotherapy without jeopardizing its toxicity.

Bevacizumab

Bevacizumab is the recombinant humanized version of a murine antihuman vascular endothelial growth factor (VEGF) monoclonal antibody A4.6.1^[62]. One randomized phase III trial utilized a regimen of irinotecan, bolus 5-FU, and FA with or without bevacizumab in patients with a good ECOG PS (PS 0 or 1)^[63]. This study demonstrated statistically significant and clinically relevant improvements in RR, TTP and survival in the bevacizumab-containing arm. Median survival was increased by 4.7 mo (15.6 *vs* 20.3 mo; $P < 0.001$). However, retrospective analyses suggested the benefit derived from irinotecan in chemotherapy combination regimens might be limited to patients with a PS of 0^[64]. Also, certain subgroups, including elderly patients, may experience significant toxicities when adding irinotecan to 5-FU/FA regimens. So, a second, supportive, placebo-controlled, randomized, phase II trial was conducted concurrently with the above-mentioned trial in patients deemed non-optimal candidates for first-line irinotecan-containing regimens^[65]. Patients had a median age ≥ 70 years, ECOG PS 0 or 1, serum albumin ≤ 35 g/dL, or prior abdominal/pelvic radiotherapy. Subjects were randomly assigned to 5-FU/FA/placebo or 5-FU/FA/bevacizumab. When compared with patients treated with 5-FU/FA alone, the addition of bevacizumab prolonged median survival by 3.7 mo, PFS by 3.7 mo, response duration by 2.4 mo, and increased the RR by 11%. Despite this higher-risk population, the regimen of 5-FU/FA/bevacizumab seemed to be well tolerated. Grade 3 hypertension was more common with bevacizumab treatment (16% *vs* 3%), but was controlled by oral medication and did not cause study drug discontinuation. No increase in grade 3 or 4 bleeding

or venous thrombotic events was seen in bevacizumab-treated patients. The authors also reported an imbalance in the incidence of arterial thrombotic events: 10% in the 5-FU/FA/bevacizumab group compared with 4.8% in the 5-FU/FA/placebo group. The more advanced age of the population may have contributed to a higher overall incidence of this adverse event.

However, additional research is needed to clarify the appropriate dosing and scheduling of various combination chemotherapy regimens (containing specifically irinotecan or oxaliplatin) plus bevacizumab in older patients.

CONCLUSION

Almost half of the CRC cases diagnosed occur in patients over the age of 70. In spite of the fact systemic chemotherapy is beneficial for patients with metastatic disease in terms of survival prolongation, symptomatic improvement and QoL, there is clear evidence that elderly patients are under-treated and under-represented or even excluded from clinical studies. Among the relevant trials for the treatment of CRC patients, probably no more than 20% of cases belong to the over 70 age-group. Nevertheless, elderly CRC patients have been shown to tolerate chemotherapy as well as younger patients in palliative settings with similar RRs. New studies are mandatory to establish particularly the safety of various combinations plus or minus biological agents in older patients. In this context, the results of a randomized phase II study evaluating the activity and safety of capecitabine in combination with oxaliplatin (CAPOX) or with irinotecan (CAPIRI) in patients ≥ 70 years could be of interest^[6]. Preliminary data from this trial confirm both combinations are active (RR 38.4% for CAPIRI and 32.2% for CAPOX) with median response durations of 8.2 mo for CAPIRI and 6 mo for CAPOX. The most frequent severe toxicities were diarrhea (CAPIRI: 19.3%; CAPOX: 14.2%) and neutropenia (CAPIRI: 22.5%; CAPOX: 2.8%). No treatment-related death occurred. These findings seem to suggest the employment of capecitabine given in doublet combination is feasible in elderly patients apart from specimen of the above-mentioned regimens.

Even though the data reported in this review have to be interpreted with caution as these results apply to patients who fulfilled the protocol requirements, age alone is not a sufficient reason to reduce the dose of drugs or to withhold adjuvant or palliative treatment from an elderly patient. The PS is probably not the best mean to estimate the conditions of elderly patients and they need more attention regarding their functional, social and mental status. The main problem which remains to be solved is the applicability of these results to all in the elderly population. Until now, specific studies on unfit older patients are very few or lacking in the literature.

APPENDIX

The information was gathered from extensive PUBMED searches (no limits to publication period were applied, but only English language papers are referenced). Additional

references, including congress abstract presentations, are included where appropriate and in particular when there are no published studies on a discussion topic.

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Crosstalk between tumor cells and microenvironment *via* Wnt pathway in colorectal cancer dissemination

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Abstract

Invasion and metastasis are the deadly face of malignant tumors. Considering the high rate of incidence and mortality of colorectal cancer, it is critical to determine the mechanisms of its dissemination. In the parallel investigation of the invasive front and tumor center area of colorectal cancer (CRC), observation of heterogeneous β -catenin distribution and epithelial-mesenchymal transition (EMT) at the invasive front suggested that there might be a crosstalk between tumor cells and the tumor microenvironment. Wnt signaling pathway is also involved in the cancer progression due to its key role in CRC tumorigenesis. Moreover, in recent years, there is increasing evidence that the regulators of microenvironment, including extracellular matrix, growth factors and inflammatory factors, are associated with the activation of Wnt pathway and the mobility of tumor cells. In this review, we will try to explain how these molecules trigger metastasis *via* the Wnt pathway.

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Key words: Invasion; Microenvironment; Colorectal cancer; Epithelial-mesenchymal transition; Wnt; β -catenin

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INTRODUCTION

Colorectal cancer (CRC) is one of the major malignancies worldwide and the second leading cause of cancer death in the United States^[1]. In the past decades, many researches in tumorigenesis and progression of CRC have focused on genes and epigenetic changes. Recently, increasing attention has been paid to cellular signal transduction in CRC, especially Wnt pathway which regulates cell growth, differentiation and death in embryogenesis and tumor development, attributing to the presence of an activating mutation of the canonical Wnt signaling pathway in about 90% of all CRCs^[2-6]. Activation of the Wnt signaling pathway is characterized by the accumulation of β -catenin in nuclei^[7]. It was reported that nuclear β -catenin is detectable in colorectal tumors and its amount is increased from early adenomas to adenocarcinomas^[8]. However, the distribution of β -catenin within an individual tumor is very heterogeneous. Immunohistochemical analysis of moderately- and well-differentiated colon adenocarcinomas reveals that accumulation of nuclear β -catenin is observed in dedifferentiated tumor cells at the invasive front and scattered in the adjacent stromal compartment. Contrarily, in central differentiated area, it is detectable on the membrane and its translocation is not found^[9,10]. Consequently, there is considerable interest in finding the means to explain such dynamic changes. Recent researches highlight the role of tumor microenvironment in cancer dissemination where cells located at the invasive front are exposed to cytokines, such as growth factors, chemokines, inflammatory factors, and extracellular matrix, which may interact with the Wnt signaling pathway resulting in the heterogeneous intracellular distribution of β -catenin^[11-13]. Therefore, this review will concentrate on the relationship between microenvironment and Wnt pathway in invasion and metastasis of CRC.

WNT PATHWAY IN CRC

The Wnt signaling pathway is involved in various differentiation events both in embryogenesis and in tumor formation when aberrantly activated. Molecular studies demonstrated that constitutive activation of Wnt/ β -catenin signaling occurs in nearly all colorectal tumors due to mutations either in *APC* gene or in less frequently β -catenin^[14,15]. Therefore, understanding the role of this pathway in CRC carcinogenesis is important.

In the absence of Wnt signaling, intracellular β -catenin levels are regulated by multiprotein complex encompass-

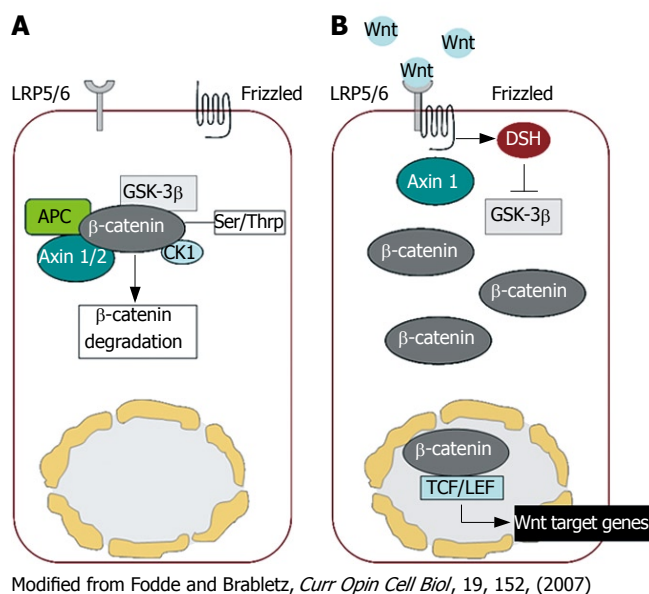


Figure 1 Schematic illustration of the canonical Wnt/β-catenin signaling pathway. **A:** In the absence of Wnt ligands, destruction complex phosphorylates β-catenin for ubiquitination and proteolytic degradation; **B:** In the presence of Wnt ligands, formation of destruction complex is not accomplished, resulting in nuclear translocation of β-catenin.

ing the adenomatous polyposis coli (APC) protein, axin, and glycogen synthase kinase 3β (GSK3β). The complex phosphorylates β-catenin making it for subsequent ubiquitination and degradation (Figure 1A). In the stimulated cells, Wnt ligands bind to one of the Wnt receptors, co-activating low-density lipoprotein receptor-related proteins (LRP). Binding of Wnts leads to phosphorylation of the cytoplasmic protein Dishevelled (Dsh) and consequently Dsh binds to axin resulting in dissociation of the complex and stabilization of β-catenin (Figure 1B). Intracellular β-catenin accumulation results in its nuclear translocation, nevertheless the molecular mechanism is still unclear. In nuclei, β-catenin works as a cofactor for transcription factors of the T-cell factor/lymphoid enhancing factor (TCF/LEF), modulating the expression of a broad spectrum of target genes (Table 1), which affects stemness, proliferation and differentiation.

In 85% familial and sporadic CRCs, the *APC* gene mutations lead to loss of β-catenin degradation of the complex function and intracellular β-catenin accumulation and translocation, which is the mark of active Wnt signaling^[4]. Accordingly, constitutive activation of this Wnt-β-catenin-TCF pathway, also called canonical Wnt pathway, is blamed for carcinogenesis in CRC.

The non-canonical Wnt pathway independent of β-catenin includes the planar-cell-polarity (PCP)-like pathway that guides cell movements during gastrulation^[14] and the Wnt/Ca²⁺ pathway^[4]. Up to now, how these pathways are involved in tumorigenesis or cancer progression is still unknown. However, there is evidence that Wnts acting through the non-canonical pathway can promote tumor progression^[16-19]. Experiments have been carried out by co-culture of breast tumor cells with macrophages, revealing that a canonical pathway in tumor cells is a necessary

Table 1 β-catenin target genes related to cancer

Function	Target gene
Cell proliferation	C-myc; Cyclin D1
Inhibition of apoptosis	MDR1/PGP; COX-2; PPARδ
Tumor progression	MMPs; uPAR, Upa; CD44; Laminin γ2; Nr-CAM
Growth factors	c-met; VEGF; WISP-1; BMP-4
Transcription factors	c-jun, fra-1; ITF-2; Id2; AF17
Negative feedback targets	Conductin; Tcf-1; Nkd

prerequisite. However, non-canonical pathway *via* Wnt5a is critical for macrophage-induced invasiveness^[19].

β-CATENIN IN CRC PROGRESSION

The capability of invasion and metastasis is the hallmark of malignant tumors. The progression of tumor cellular dissemination leading to invasive growth includes the detachment from primary cancer, migration, access to blood or lymphatic vessels and development of secondary tumors. Cellular dissemination is characterized by disordered cell-cell interactions and cell adhesion. Disintegration of cell adhesion molecules, especially β-catenin, has been implicated in this process. However, only β-catenin in the membranes, a stable subcellular localization, forms an adherent complex with α-catenin and E-cadherin which is regulated by tyrosine phosphorylation. Phosphorylated β-catenin is dissociated from the adherent complex and transferred to the cytoplasm, where β-catenin can be degraded or translocated into nuclei, triggering dysregulation of Wnt pathway. Importantly, cooperative effects on tumor development of defects in E-cadherin-mediated cell adhesion and activation of β-catenin-mediated signal transduction are observed in human CRC^[20]. Moreover, a tissue microarray-based analysis of a large number of cases, performed by Lugli *et al*^[21] demonstrated that increased nuclear β-catenin expression and loss of membranous E-cadherin are two independent, adverse prognostic factors in sporadic CRC, suggesting that the role of β-catenin in tumor invasion and metastasis is not just attributed to interaction with E-cadherin, therefore other mechanisms may be involved, such as Wnt/β-catenin signaling pathway.

Furthermore, as the downstream effector of canonical Wnt pathway, nuclear β-catenin cooperating with TCF/LEF initiates expression of target genes (Table 1), some of which can improve tumor progression. MMP-7, a target of β-catenin/TCF signaling, is expressed in up to 90% of CRCs and its expression in the invasive front as well as in urokinase plasminogen activator (uPA) and urokinase plasminogen activator receptor (uPAR) is related to unfavorable outcome in CRC^[22,23]. Fascin, a novel target of β-catenin/TCF signaling, is expressed at the invasive front of human colon cancer, suggesting that it plays a potential role in the development of colon cancer metastasis^[24]. It was reported that intratumorous heterogeneity in CRC correlates with differential expression of 510 genes between the central tumor region and the invasive front, isolated by laser-microdissection in the same tumor samples^[24]. This *in vivo* analysis shows over-expression of known Wnt/β-catenin target genes either in the entire

tumor tissue or specifically at the invasive front. Whether these target genes expressed at the front are involved in the tumor invasive process still needs to be further studied. Furthermore, the concomitant high expression in 2 groups of Wnt/ β -catenin target genes, inflammation- and tissue repair-related genes, at the invasive front supports the hypothesis that inflammation-activated microenvironment may trigger selective Wnt/ β -catenin target gene expression and contribute to the progression of CRC^[25]. Accordingly, similar in tumor initiation, Wnt pathway activation (detectable by nuclear accumulation of β -catenin and expression of some target genes) might be functionally associated with cancer dissemination.

In modestly- and well-differentiated tumor, membranous expression of β -catenin in tumor center retains whereas nuclear β -catenin is observed in dedifferentiated tumor cells localized in the invasive area^[10]. Since tumor cells in an individual tumor harbor APC mutations, this alteration alone cannot lead to the heterogeneous distribution of β -catenin, but its translocation has to be explained by additional events^[26]. Whether nuclear β -catenin accumulation is the sign of motility enhancement of tumor cells and what initiates β -catenin heterogeneous distribution, are two questions arising from these observations.

EPITHELIAL-MESENCHYMAL TRANSITION IN CRC DEVELOPMENT

In the majority of sporadic CRCs, well-, modestly-, and well-differentiated adenocarcinomas, tumor cells at the invasive front lose their epithelial characteristics and take on the properties that are typical of mesenchymal cells, which require complex changes in cell architecture and behavior. Such transition from epithelial- to mesenchymal- cells, dubbed as epithelial-mesenchymal transition (EMT), is considered a fundamental event in the metastatic cascade. The essential features of it are the disruption of intercellular contacts and the enhancement of cell motility, thereby leading to release of cells from the parent epithelial tissue. The resulting phenotype is suitable for migration and, thus, for tumor invasion and dissemination, allowing metastasis progression to proceed. Although the molecular bases of EMT have not been completely elucidated, several interconnected transduction pathways and a number of potentially involved signaling molecules, including β -catenin, have been identified^[27,28].

Activated β -catenin is directly linked to EMT. The activation of Wnt signal pathway results in the activation of β -catenin/TCF transcriptional regulators such as snail^[29,30] and slug^[31], which regulate the changes in gene-expression patterns underlying EMT. Similarly, in the study of breast cancer cells, Yook *et al* demonstrated that canonical Wnt pathway engages tumor cell dedifferentiation and tissue-invasive ability through an axin-2-dependent pathway to identify a new mechanistic β -catenin-TCF-regulated axin2-GSK3 β -Snail1 axis, thus gaining insight into cancer-associated EMT program^[32]. It was reported that Wnt/ β -catenin signaling pathway plays a pivotal role either in gastric cancer formation or in tumor invasion and dissemination^[33]. In cell culture experiments, cells with β -catenin activation

lose their polarity and disrupt cell-cell contacts and EMT morphologically^[34,35]. Moreover, immunohistochemical stains demonstrate alternations of the actin cytoskeleton in these cells, indicating that nuclear β -catenin accumulation is functionally related to EMT in budding tumor cells at the tumor-host interface.

MICROENVIRONMENTAL REGULATION IN β -CATENIN TRANSLOCATION AND EMT INDUCTION

The dynamic changes in the above non-random distribution of β -catenin and EMT of tumor cells at the invasive front of CRC, can be at least partially explained by interactions with the tumor environment. A micro-ecosystem exists at the invasive front of tumor where the stromal cells interact with parenchymal cells by producing extracellular matrix and secreting cytokines that directly or indirectly promote cell invasion^[14,36]. Moreover, it also appears that inflammatory cells are involved in the formation of tumor metastasis^[25,37].

Epithelial-mesenchymal interactions are essential for intestinal development. Thus, more investigations should be focused on mesenchymal factors, particularly the components of extracellular matrix, because they have a potent regulatory effect on tumor cells. Recent studies demonstrated that Wnt ligands are expressed in both mesenchymal and epithelial cells of the colon^[38]. It was also reported that local regulation by Wnt signals of diverse cell signaling pathways in fibroblasts could have multifaceted consequences for tissue microenvironments *in vivo*, including the balance between cell differentiation and proliferation, as well as between cell migration and adhesion^[36]. Mesenchymal forkhead transcription factors, *Foxf1* and *Foxf2*, can limit paracrine Wnt signaling and promote extracellular matrix production in gut, and deletion of *Foxf1* and *Foxf2* is accompanied with increased mesenchymal expression of Wnt5a and β -catenin nuclear accumulation in epithelial cells, indicating that there is a crosstalk between stromal cells and parenchymal cells involving Wnt signaling^[39]. There are extensive data to support the relation between extracellular matrix and signal pathway in tumorigenesis. Tsuboi K *et al*^[40] investigated the relationship of galectin-3 expression, a component of extracellular matrix, to the clinicopathological factors, and found that reduced galectin-3 expression is related to invasion and metastasis of CRC. In contrast to β -catenin, the expression of galectin-3 is lower at the invasive front of a tumor. Whether β -catenin regulates galectin-3 expression or other signaling pathways are involved in the process is still controversial.

In addition, cell culture experiments have also revealed a role of cytokines, such as growth factors, in the intracellular β -catenin distribution, as well as in the induction of EMT^[41]. One of the related growth factors is the hepatocyte growth factor (HGF), which is found in CRC. It was reported that HGFR and β -catenin physically interact in a complex, which is disassembled after HGF treatment^[42]. Moreover, HGF treatment promotes β -catenin/TCF transcriptional activity in CRC cells. HGF also stimulates

cells leading to cell scattering. Therefore, a self-amplifying positive feedback loop between HGFR and β -catenin in CRC promotes tumor growth and invasion^[42]. Like HGF, Platelet-derived growth factor (PDGF) also can activate EMT in CRC cells by enhancing Wnt signaling. A recent study has shown a novel Wnt-independent pathway that enhances β -catenin signaling to nuclei^[12]. PDGF promotes tyrosine phosphorylation of p68, which binds to β -catenin and inhibits its phosphorylation by GSK3 β ^[12]. A new EMT pathway from PDGF and another route to nuclear β -catenin signaling have been identified^[43]. Similarly, the epidermal growth factor (EGF) and transforming growth factor β (TGF β) can also enhance Wnt/ β -catenin signaling by phosphorylating p68^[12].

It has been widely accepted that inflammatory cells in colorectal tumors are associated with the progression to malignancy. Brown *et al*^[44] reported that non-steroidal anti-inflammatory drugs (NSAIDs) can decrease the number and size of intestinal polyps in Apc-mutation mice by inhibiting cyclooxygenase-2 (COX-2), one of the main enzymes in prostaglandin biosynthesis. To investigate the mechanism, a recent study by Castellone and collaborators^[37] indicate that COX-2 and its proinflammatory metabolite prostaglandin E2 (PGE2) enhance colon cancer progression *via* its heterotrimeric guanine nucleotide-binding protein (G protein)-coupled receptor, EP2. This signaling route involves the activation of PI3K and protein kinase A α by free G protein and is directly associated with the G protein signaling (RGS) domain of axin, thus leading to GSK3 β inactivation, relief of inhibitory phosphorylation of β -catenin and activation of Wnt signaling pathway^[37]. Therefore, these findings suggest that COX-2 and inflammation can promote the progression of colon cancer. It was recently reported that co-culture of tumor cells and macrophages leads to up-regulation of Wnt5a in the latter and that non-canonical signaling *via* Wnt5a in cancer cells is critical for invasion^[19]. However, whether a similar interaction between cancer cells and tumor-associated macrophages occurs in CRC is still unknown.

SUMMARY

Since tumor cells at the invasive front display nuclear accumulation of β -catenin and EMT features associated with local activation of Wnt signaling pathway, dissemination of cancer cells is not due to gene mutation alone. The importance of tumor microenvironment where extracellular matrix, growth factors and inflammatory factors play a key role in tumor invasion cannot be overlooked. A complex network, which is orchestrated by Wnt pathway and other signaling pathways, may be involved in the regulation of tumor-microenvironment crosstalk. Further study is needed to investigate the specific role of tumor cells and the microenvironment of tumor in invasiveness. Although recent researches have illuminated the involvement of Wnt pathway in cancer development, a more comprehensive view of how cancer spreads will likely emerge in the future, allowing us to provide new potential therapeutic targets for the treatment of aggressive and recurrent CRC in clinical practice.

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ESOPHAGEAL CANCER

Comparative genomic hybridization analysis of genetic aberrations associated with development of esophageal squamous cell carcinoma in Henan, China

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Abstract

AIM: To characterize cytogenetic alterations in esophageal squamous cell carcinoma (ESCC) and its metastasis.

METHODS: A total of 37 cases of primary ESCC and 15 pairs of primary ESCC tumors and their matched metastatic lymph nodes cases were enrolled from Linzhou, the high incidence area for ESCC in Henan, northern China. The comparative genomic hybridization (CGH) was applied to determine the chromosomal aberrations on the DNA extracted from the frozen ESCC and metastatic lymph node samples from these patients.

RESULTS: CGH showed chromosomal aberrations in all the cases. In 37 cases of primary ESCC, chromosomal profile of DNA copy number was characterized by frequently detected gains at 8q (29/37, 78%), 3q (24/37, 65%), 5p (19/37, 51%); and frequently detected losses at 3p (21/37, 57%), 8p and 9q (14/37, 38%). In 15 pairs of primary ESCC tumors and their matched metastatic lymph node cases, the majority of the chromosomal aberrations in both primary tumor and metastatic lymph node lesions were consistent with the primary ESCC cases, but new candidate regions of interest were also detected. The most significant finding is the gains of chromosome 6p with a minimum high-level amplification region at 6p12-6q12 in 7 metastatic lymph nodes but

only in 2 corresponding primary tumors ($P = 0.05$) and 20p with a minimum high-level amplification region at 20p12 in 11 metastatic lymph nodes but only in 5 corresponding primary tumors ($P < 0.05$). Another interesting finding is the loss of chromosome 10p and 10q in 8 and 7 metastatic lymph nodes but only in 2 corresponding primary tumors ($P < 0.05$).

CONCLUSION: Using the CGH technique to detect chromosomal aberrations in both the primary tumor and its metastatic lymph nodes of ESCC, gains of 8q, 3q and 5p and loss of 3p, 8p, 9q and 13q were specifically implicated in ESCC in Linzhou population. Gains of 6p and 20p and loss of 10pq may contribute to the lymph node metastasis of ESCC. These findings suggest that the gains and losses of chromosomal regions may contain ESCC-related oncogenes and tumor suppressor genes and provide important theoretic information for identifying and cloning novel ESCC-related oncogenes and tumor suppressor genes.

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Key words: Comparative genomic hybridization; Genetic alterations; Esophageal squamous cell carcinoma; Metastatic lymph nodes

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INTRODUCTION

Advances in diagnostic and therapeutic modalities for malignancies have improved the survival of cancer patients. However, the mortality rate of patients with esophageal squamous cell carcinoma (ESCC) is still very high due to its highly invasive nature and potential to metastasize to lymph nodes and distant organs. ESCC is one of the leading causes of cancer-related death

in Linxian, Henan Province in northern China, with a mortality rate of 161/100 000 for male and 103/100 000 for female. Recent studies by us and other authors have indicated multiple genetic alterations underlying the multistage carcinogenesis of ESCC, such as p53-Rb pathway^[1]. However, the mechanisms of human esophageal multistage carcinogenesis in this area, especially the difference in genetic changes between primary ESCC and lymph node metastasis, are largely unknown.

Comparative genomic hybridization (CGH) analysis can provide comprehensive information about relative chromosomal losses and gains in malignant tumors *in vitro*^[2]. This technique can detect the changes of recurrent copy number and may highlight chromosomal regions containing genes that contribute to cancer development and/or progression. In this study, we used CGH analysis to examine 37 primary ESCC and 15 pairs of primary ESCC tumors and their corresponding metastatic lymph nodes, to elucidate the genetic pathway of carcinogenesis and to clarify the metastatic mechanisms of genetic aberrations in ESCC.

MATERIALS AND METHODS

The 37 primary tumor samples and 15 pairs of primary ESCC and their matched lymph node metastasis in this study were all from Linzhou, Henan, the high incidence area for ESCC. The primary specimen for each patient was collected at the time of surgical resection at the Department of Surgery, Yiaochun Hospital and Linzhou Hospital. All the patients underwent esophagectomy without preoperative radiotherapy and/or chemotherapy. Tumor tissue specimens were frozen in liquid nitrogen and kept in a freezer at -80°C until use.

Microdissection and DNA extraction

Tumor tissue was selected by histopathologic examination on the basis of estimated more than 80% cancer cells. The metastatic lymph nodes were embedded in OTC and cryosected into 15 µm serial slides under -20°C. For DNA extraction, we cut 22 serial 15 µm sections. The first and last ones were used for hematoxylin-eosin (HE) staining, and the remain 20 were lightly stained with hematoxylin. Under microscopic (MZ 12, Leica, Bensheim, Germany) observation, tumor tissues were microdissected manually from surrounding stromal tissues and normal cells with a disposable fine needle, and tissue fragments were collected and transferred into a microtube.

Genomic DNA was extracted from tumor specimens by proteinase K/sodium dodecyl sulfate digestion followed by phenol/chloroform/alcohol extraction. Normal reference DNA was prepared from peripheral blood lymphocytes of healthy donors.

Slide preparation

Metaphase chromosome spreads were prepared from peripheral blood leukocytes of healthy donors. Blood cells were cultured for 72 h in RPMI1640 containing 15% fetal bovine serum and penicillin-streptomycin (PHA 5 µg/mL). Blood cells were harvested by arresting with Colcemid

(0.05 g/L) for 1h, followed by hypotonic treatment in KCl (0.075 mmol/L) for 20 min on ice and fixation in cold methanol: acetic acid (3:1).

Comparative genomic hybridization(CGH)

CGH was performed essentially as described previously^[2]. Briefly, genomic DNA from a tumor sample and a sex-matched normal reference was labeled directly with Spectrum Green-dUTP and SpectrumRed-dUTP (Vysis, Downers Grove, IL, USA) by nick translation. Two hundred nanograms of labeled tumor DNA and normal DNA probes were used in a 10 µL hybridization mixture (containing 55% formamide, 2 × SSC), and 10 µg human CotI DNA, which was denatured at 75°C for 5 min. The slide containing normal metaphase spreads was treated with RNase (100 mg/L) at 37°C for 1 h and then denatured at 75°C in 70% formamide, and 2 × SSC for 5 min. Hybridization with probes was then carried out at 37°C in a moist chamber for 72 h. The slide was then washed in 0.4 × SSC/0.3% NP-40 at 75°C for 2 min and then in 2 × SSC/0.1% NP-40 at room temperature for 2 min. After washing, the slide was counterstained with 1 mg/L DAPI in an antifade solution.

Digital image analysis

The hybridized metaphase chromosomes were analyzed using a digital image analysis system containing a Zeiss Axiophot microscope equipped with a Metachrome II cool-charged device camera (Photometrics, AZ). Three images of each metaphase were captured using filter wheel-mounted, single band excitation Rhodamine, FITC, and DAPI filters. The image analyses were carried out using Quips CGH Analysis software (Vysis). Five metaphases were analyzed to generate fluorescence ratio profiles in each case. Interpretation of the profiles was performed according to the program guidelines. The thresholds used for interpretation of gains and losses of a DNA sequence copy number was defined as a tumor/reference ratio greater than 1.25 or less than 0.75, respectively, by both the standard and the reverse hybridization methods.

Statistical analysis

We analyzed the genetic aberrations of ESCC using the Fisher exact test for independence. Differences with a *P* value less than 0.05 were considered statistically significant.

RESULTS

CGH analysis

A total of 230 DNA copy number gains and 212 DNA copy number losses were found in 37 ESCC samples, with an average of DNA copy number gains of 6.22 and DNA copy number losses of 5.73 per patient. In ESCC, the gain was most frequently detected on chromosome arms 8q (29/37, 78%), 3q (24/37, 65%) and 5p (19/37, 51%), (the chromosomal aberrant frequency on chromosome 6q, 7p and 7q was similar, all 38%), which was followed by (from high to low) 18p (12/37, 32%), 1q (11/37, 30%), 11q (10/37, 27%), 20q (10/37, 27%), 12p (9/37, 24%), 13q (6/37, 16%) and 18q (6/37, 16%). There were 20 cases

Table 1 CGH results of 37 ESCC cases, Linzhou, Henan

Case No.	Gains	Losses
66908	5p, 10q11-10q21	3p21-3p11, 9,10q23-10qter, 15
66865	1p31-1qter, 3q, 3q22-3qter, 5p, 6q15-6qter, 7p14-7qter, 8q13-qter, 18p	4pter-4p13, 9q, 5q11-14, 11p, 18q12-18qter
66867	3q, 5pter-5q12, 6p12-6q12, 7p12-7qter, 8q, 16	3pter-3p13, 4q23-4qter, 8p, 9,11p13-11qter, 13,17p, Xp
66909	3q, 5p, 7,8q12-8qter,	9p13-9qter, 19
66755	1q, 2p, 3q, 8q, 9p, 11q13-11q21	1pter-1p31, 3pter-3p13, 8p, 11q22-11qter, 15,16,17,19,21,22
16182	4p13-4qter, 8p12-8qter, 9p, 16p11-16qter, 16p11-16q21, 18p	1pter-1p32, 3p, 4pter-p14, 11q22-11qter, 18q, 19
66907	2q14.1-14.3, 2q31-32, 3q23-3qter, 5p, 6q, 7p, 8,12p, 18p	3p, 4,5q, 10,11q21-qter, 12q, 17p, Xp
66912	2q14-2qter, 3q22-3qter, 5p, 6p12-6q15, 8q, 12q11-12q22, 13,20q	1pter-1p31, 5q, 11pter-11q12, 16,17p, 19
66910	6p21-6q13, 7q11-7q31, 8q, 13	1pter-1p31, 8p, 16q
66948	2p, 3q, 3q22-3qter, 7,8q, 10p, 14,16q11-12	1pter-1p31, 3p, 6q, 8p, 11q14-11qter, 13,19,21
16146	3q, 5pter-5q12, 6p21-6q12, 7q21-7q22, 8q, 17q, 18pter-18q11	8p, 9q, 16,18q12-18qter
16634	3p12-3qter, 5pter-5q12, 7q21-7qter, 8q, 12p, 18p, 20,20p	1pter-1p33, 4pter-4p15, 9,11pter-11q12, 13,16q, 17,18q, 19p, 21,22
16179	1q, 3q, 3q22-3qter, 4p13-4q21, 5p, 6p, 7, 8q, 13	1pter-1p31, 3p, 5q, 8p, 9,10,14,19,Y
16601	5p, 8p12-8qter,Y	3p, 19
16605	6p, 8q, 11q11-11q21, 14,16pter-16q22, 17p, 20p11-20q13, 22	11q22-11qter, 18q12-18qter
16609	1q, 2,3q, 3q22-3qter, 5p, 8q, 11p13-11q21, 12p, 13q21-13qter, 16q12-16qter	1p, 3p, 4,5q, 8p, 9p, 10,12q, 18p11-18qter, 21, Xp
16610	3q, 7p, 8q, 12p12-12q23, 16p11-16q21, 18	1pter-1p31, 8pter-8p12, 11,17,19,Y
16615	1q31-1qter, 2pter-2q33, 3q13-3qter, 4q12-4q21, 5p, 7q21-7q31, 8q22-8qter, 12,18p	2q34-2qter, 5q13-5q14, 7p11-7q11, 8p, 9,22, X
16616	6p21-6qter, 7pter-7q21, 8p12-8qter, 10,14	7q22-7qter
16629	1p32-1p21, 2q11-2q32, 3q22-3qter, 6p, 8q, 9p, 11p, 12q14-12q22, 18,20	2q33-2qter, 6q, 8p, 9q, 10q, 11q, 13,16, X
16639	1p31-1p13, 3q13-3qter, 11q13-q23, 12pter-12q13, 14	3pter-13, 4pter-12, 5q21-qter, 21,22, Xpter-q13
16658	3q, 4pter-4q13, 5p, 6p12-6q14, 8q	3p, 8p, 9q21-9qter, 11,19
16721	1p11-1q22, 7pter-7q31, 14q21-14q23, 19,20	2p, 3p, 5q12-5qter, 6q, 11p, Y
16740	5p13-5q13, 18pter-18q12	6pter-6p21, 14q13-14qter
19110	1q21-1qter, 2q, 6,7p, 11p, 11q13-q22, 14, 16p13-16p11	1pter-1p34, 8pter-8q12, 8q23-8qter, 15q22-15qter, 16q, 17, 22
19315	6p12-6q14, 13,16q11-16q21, 18pter-18q12	1p13-22, 3pter-q21, 8pter-12, 18q21-qter, X
19419	2q24-2q32, 3q, 8,13,20	3p, 9p22-9q21, 10,16p, 17
16172	8q22-8qter	13
16181	3q13-3qter, 7,8,12,17,18p, 20,22	2q14-2q34, 3p, 4q, 5q11-q23, 6q11-24, 13
16186	3p12-3qter, 5p, 6p21-6q15, 7, 8q, 11p14-11q14, 16,17pter-17q12, 20pter-20q11	13,18q
16604	1p31-1qter, 3q, 4p12-4q12, 6, 7,8p22-8qter, 10pter-10q11, 11q12-11q23, 14q11-14q31	2pter-2q22, 3p, 5q21-5qter, 9p12-9qter, 13,15,17,18
16624	1pter-1p31, 3pter-3p21, 3q, 5p, 6p21-6p12, 7p, 8q, 11,18pter-18q12, 19p13-19qter, 20	2p, 3p21-3p11, 4,5q, 8p, 10,14,16q, Y
16633	8q22-23, 9,12p11-12q21, 21,X	3p, 6,10,11p, 12q21-12qter, 16q23-16qter, 18q,
16632	1q, 2pter-q32, 3p12-qter, 5pter-q12, 8p22-qter, 9q, 11, 12pter-q13, 15, 16p12-q21, 17p12-q24, 18pter-q12	3pter-3p13, 4,10,13
16624	3q, 3q22-3qter, 5p, 11p11-11q13	3pter-3p12, 11q14-11qter, 13,18, X
16625	1q, 3q, 4p14-4q21, 4q26-4qter, 19p11-19q13	1pter-1p319q33-9qter, 15,16,17p
66945	1p31-1qter, 3q, 5p, 6q14-6qter, 8q12-8qter, 9pter-9q31	3p, 4,5q, 9q31-9qter, 11,13,14

with high copy number amplifications (tumor/reference ratio > 1.5), which were located on chromosome 1pter-1p31, 1p11-1q22, 3q22-qter, 4p13-4q31, 5p, 6p12-6q14, 8q, 9p, 11q12-11q23, 11q13-11q23, 16p11-16q21, 18pter-18q12, 18p and 20p. The chromosomal profile of DNA copy number losses was characterized as follows: the most frequently detected loss was on 3p (21/37, 57%), which was followed in turn (from high to low) by 8p (14/37, 38%), 9q (14/37, 38%), 11q (13/37, 35%), 13q (13/37, 35%), 5q (12/37, 32%), 1p (12/37, 32%), 4p (11/37, 30%), 18q (11/37, 30%), 16q (10/37, 27%), 17p (10/37, 27%) and 19p (10/37, 27%). A summary of genetic aberrations detected by CGH in ESCC is provided in Table 1 and Figure 1.

Differences in copy number alterations between 15 pairs of primary ESCC and their matched lymph node metastasis

A total of 102 gains and 90 losses were found in 15 primary ESCC samples, with an average of gains and losses per patient of 6.8 and 6.0, respectively, and 110 gains and 142 losses were found in 15 lymph nodes metastasis samples, with an average of gains and losses per patient

of 7.4 and 9.5. More genetic changes (252) were found in lymph nodes metastasis than in the corresponding primary lesions (192), especially the losses (9.5/case *vs* 6.0/case). The copy-number changes for the entire genome detected in these 15 primary ESCC and their corresponding metastatic lesions are summarized in Table 2 and Figure 2. In primary tumors, the most frequently detected sites of chromosome gain were 3q (15 of 15, 100%), 8q (11 of 15, 73%), 1q (11 of 15, 73%), 12p (8 of 15, 53%), 18p (6 of 15, 40%), 5p (7 of 15, 47%), 6p (7 of 15, 47%), 20p (11 of 15, 73%). In metastatic lymph nodes, the most frequently detected sites of chromosome gain were 3q (14 of 15, 93%), 8q (10 of 15, 67%), 1q (11 of 15, 73%), 12p (8 of 15, 53%), 18p (6 of 15, 40%), 5p (7 of 15, 47%), 6p (7 of 15, 47%), 20p (11 of 15, 73%). High copy-number amplification with a minimum gain 3q24-qter was detected in two primary tumors and six metastatic lymph nodes. The other three high copy-number amplifications of 6p21-6q12 were detected only in metastatic lymph nodes but not in their corresponding primary tumors. The high copy-number amplification of 20p was detected in metastatic lymph node lesions but not in their corresponding primary tumor. In primary tumors, the chromosomal profile of

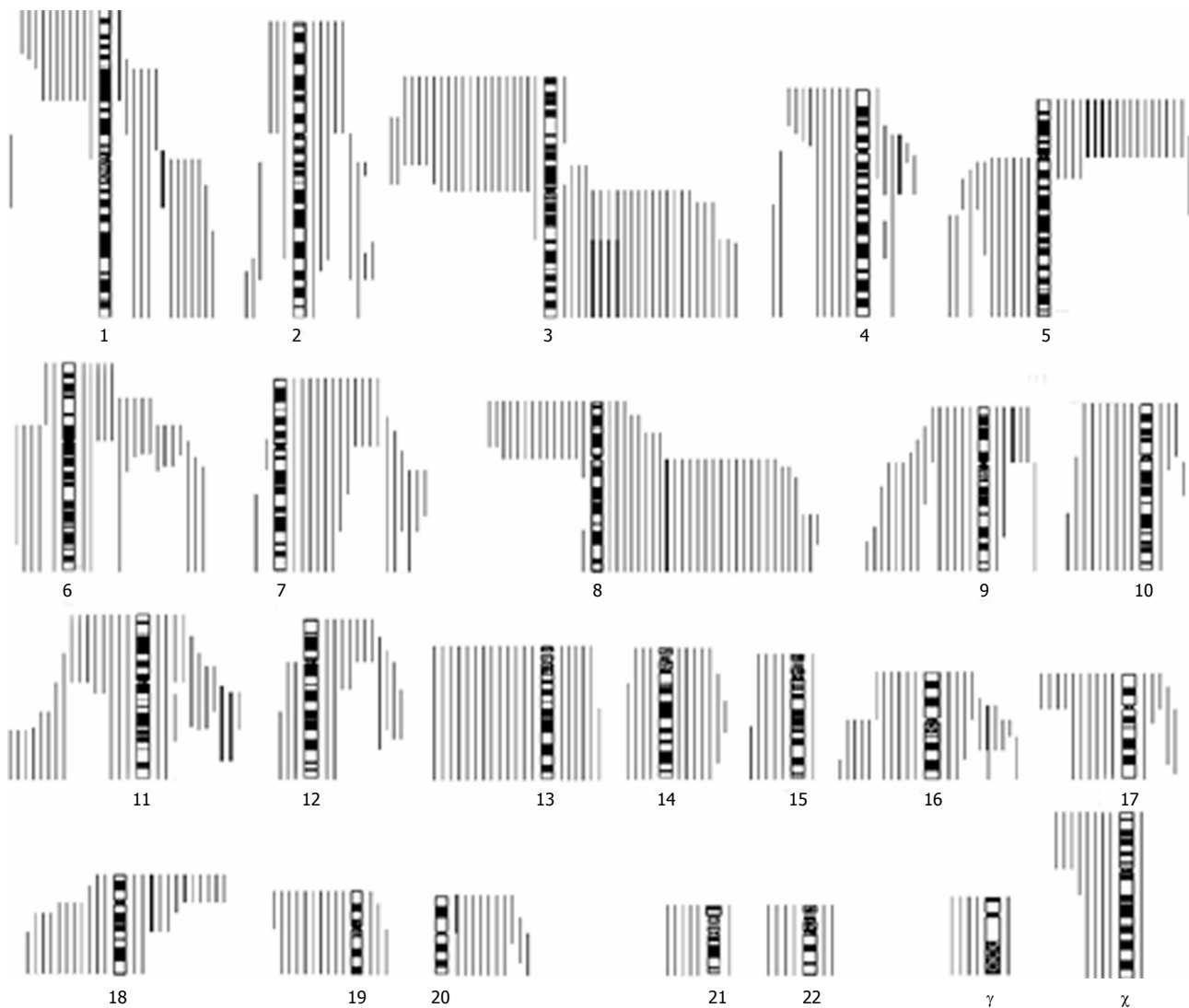


Figure 1 Copy number alterations of 37 ESCC cases analyzed by CGH. Chromosomal regions of gain are on the right side of the chromosome ideograms, and regions of loss are on the left side.

DNA copy number losses were 3p (10 of 15, 67%), 10p (2 of 15, 13%), 10q (2 of 15, 13%), 4q (6 of 15, 40%), 4p (7 of 15, 47%), 19p (8 of 15, 53%), 13q (4 of 15), 1p (7 of 15), 17p and 18q (4 of 15, 27%). In metastatic lymph nodes, the chromosomal profiles of DNA copy number losses were 3p (12 of 15, 80%), 10p (8 of 15, 53%), 10q (7 of 15, 47%), 4q (10 of 15, 67%), 4p (9 of 15, 60%), 19p (10 of 15, 67%), 13q (8 of 15, 53%), 1p (7 of 15, 47%), 17p and 18q (7 of 15, 47%).

Statistically, in DNA copy number gains on chromosome 6p and 20p, there were significant differences between primary ESCC lesions and their corresponding lymph nodes ($P < 0.05$). In DNA copy number losses on chromosome 10pq, differences were significant between primary ESCC lesions and their corresponding lymph nodes ($P < 0.05$), (Table 3).

DISCUSSION

We have identified a genome-wide map of genetic alterations in ESCC, and compared primary tumor and metastatic lymph nodes in this study. The majority of the

chromosomal aberrations in both primary tumor and metastatic lymph node lesions were consistent with the previous reports. For example, the gains of 3q, 5p, 8q23-ter and 20q and deletions of 3p25, 4p, 6q21, 9q22.3-q31, 9p, 11q22-qter, 13q12-13, 18q22.3, and 19q had been detected as frequent chromosomal alterations in ESCC in at least one of the previous CGH studies^[3-6]. By comparing the primary ESCC with metastatic lymph nodes, we did detect new candidate regions of interest, such as 6p, 20p and 10pq, which may harbor the genes involved in lymph node metastasis.

The highest frequency of DNA gain in ESCC occurred at chromosome 8q (78%), the chromosomal region at 8q harbors MYC gene (8q24.1) which has been identified with a high frequency of amplification in ESCC^[7,8]. Gains of 3q were commonly seen in ESCC^[9]. Possible candidate genes involved in tumor development include the genes for ribosomal protein L22 (RPL22), butyrylcholinesterase (BCHE), glucose transporter 2 (SLC2A2), transferring receptor (TFRC), thrombopoietin (THPO) and the phosphatidylinositol-3 kinase catalytic α -polypeptide (PIK3CA). Deletion of 9p and gain of 5p were seen

Table 2 CGH results of lymph node metastasis, Linzhou, Henan

Case No.	Gains	Losses
16148	2p12-2q32, 3p12-3qter, 5p, 9q	3pter-3p13, 5q31-5qter,X
L16148	1q, 2q11-2q31, 3q, 5p, 9p12-9qter, 12,20	2q32-2qter, 3p, 4,5q, 6pter-6p22, 19,X
16668	3q13-3qter, 4q31-4qter, 7pter-1p11, 8q12-8qter, 18p	1p, 4pter-4p14, 4q21-4q28, 7q22-7qter, 9p
L16668	3q, 4p12-4q13, 7p15-7q22, 8p21-8qter, 13,15pter-15q15, 18pter-18q12, 20pter-20q12	3p, 4pter-4p13, 4q13-4q25, 7q31-7qter, 9p, 11q13-11qter
16669	3p12-3qter, 7p, 8q, 12pter-12q13, 14	1p, 3pter-3p12, 4p, 10,11,17,19
L16669	3p12-3qter, 6p12-6q14, 7p, 8p12-8qter, 14,20p11-20qter	1p, 3pter-3p12, 4p, 10,11,19
16v688	1q21-1qter, 3q, 5p, 6p12-6q24, 7q11-31, 8p22-8q23, 11q, 11q11-11q14, 18pter-18q12	3p, 4q13-4qter, 6pter-6p21, 7q32-7qter, 9p12-9qter, 10p, 11p, 16q, 17pter-17p11, 17q22-17qter, 19
L16688	1p13-1qter, 1p13-1q21, 2,3q, 6p21-6q12, 8q11-8q23, 11q, 11q11-11q14, 12p, 18pter-18q11, 18p11-18q11, 20	3p, 4,5pter-5q15, 6pter-6p21, 6q16-6qter, 7p, 7q31-7qter, 8pter-8p12, 9q, 10p, 11p, 13,17p, 17q22-17qter, 19,21
16691	1q, 2pter-2q21, 3q, 11q11-11q13, 13q14-13qter, 16q11-16q13, 17q21.1-21.3, 20	3p, 8q11-8q22, 9q, 11q14-11qter, 13pter-13q14, 16p, 19,21,22
L16691	1q, 2pter-2q21, 3q, 11q11-11q13, 13q14.3-13qter, 16q11-16q13, 17q21.1-17q21.3, 20	1pter-1p32, 3p, 8q11-8q22, 9q, 11q14-11qter, 13pter-13q14.2, 16p, 17p, 19,21,22
16670	3q, 8q, 12p, 14,16p11-16q21, 18p, 20	1pter-1p31, 9p12-9qter, 17,18q, 19,Y
L16670	3q, 8q, 12p, 14,18p, 20	1pter-1p34, 3pter-3p13, 9q, 10,11p11-11q12, 17,19p,Y
16710	1p33-1qter, 2pter-2q32, 3p13-3qter, 5pter-5q12, 6q16-6qter, 7p, 11q11-11q23, 12pter-12q15, 13,16p13-16qter, 17p,	4p, 18pter-18q21
L16710	1q, 2p22-12, 2q22-2q32, 3q, 5p, 6q21-6qter, 7p, 9pter-9p21, 11q11-11q14, 13,16q, 17pter-17q12	1p, 4,6pter-6q16, 8pter-8p12, 10pter-10p12, 10q23-10qter, 11p, 11q14-11qter, 12q, 16p, 18,21
16727	1p13-1q24, 2p24-2p23, 2p14-2p12, 3q13-3qter, 5p, 7q11-7qter, 8q, 12pter-12q12, 14q23-14qter, 18pter-18q12, 20	1p, 2q32-2qter, 3pter-3p21, 4,5q, 8p, 11p, 18q12-qter
L16727	1q, 2p14-2p11, 3q13-3qter, 5p, 7q11-7qter, 8q, 12pter-12q13, 14,17q, 18p, 20p	1p, 2q32-2qter, 3p, 4,5q, 9p, 10p, 11p, 15pter-15q22, 18q12-18qter, 21
16728	3q, 8p12-8qter, 11p11-11q14, 12pter-12q12, 16p, 18pter-18q12, 20	3p,Xp
L16728	1p21-1pter, 3q, 3q24-3qter, 7p12-7q22, 8q, 8q21-8qter, 12p12-12q12, 14q11-14q21, 20p	4q, 8pter-8p22, 10,11,13q12-13q31, 14q23-14qter, 20q,Xp
16730	1q, 3q, 6,7,8,12pter-12q13, 20q12-20qter	3p, 9p, 16q, 19p13-19q13
L16730	1q, 2,3q, 6,6p21-6q13, 12pter-12q14, 18p11-18q12	1p, 3p, 4q26-4qter, 5q, 9,10,13,16,18q21-18qter, 19p, 21
16731	3q, 4p13-4q13, 16q11-16q12, 11p13-11q13, 12	9,13
L16731	3q, 4p13-4q13, 8p12-8qter	3p21-3p11, 9q, 10,17,18q12-18qter, 19
16738	1q, 3p13-3qter, 5p, 8q	1pter-1p31, 3pter-3p14, 4,11,16,17,19,Xpter-q21
L16738	1p13-1qter, 1p13-1q21, 5pter-5q12, 6p21-6q14, 8q, 20	1pter-1q13, 2pter-2p21, 3p, 4,8p, 11pter-11p12, 11q14-11qter, 13,16p, 17,18, Xpter-q21
16742	1q11-1q24, 3q, 3q24-3qter, 5pter-5q12, 8p11-8qter, 9q, 10p, 12p, 19p12-19q12, 20	1p, 3p, 4,6q16-6qter, 8pter-8p12, 9p, 10q22-10qter, 11,13,14q21-14qter
L16742	1q11-1q24, 2p21-2qter, 3q, 3q22-3qter, 5p13-5q13, 7,8q, 9q21-9q32, 10p12-10q11, 12pter-12q13, 20	1qter-1q32, 3p, 4,6q, 8p, 10q21-10qter, 11,12q21-12qter, 13,14q21-14qter, 19
16745	1q, 2q24-2q33, 3q, 5p, 6q13-6q24, 8,9p, 10pter-10q21, 12q14-12q21	1pter-1p32, 2q21-2q24, 2q34-2qter, 3p, 4,5q, 7q31-7qter, 11q14-11qter, 13pter-13q21, 16,18,19,21
L16745	1q, 2q24-2q35, 3q, 5p, 6p21-6q25, 8	2q21-2q24, 2q34-2qter, 3p, 4,5q, 7q31-7qter, 11q14-11qter, 13pter-13q21, 16,18,19,21
16770	3p12-3qter, 8p12-8qter	17p, 19p, 22
L16770	3q, 6p, 16p11-16q21, 18p, 20	11,13,17p, 18q, 19p, 22

commonly in CGH studies of patients with ESCC^[3-5,9] and both are related to the progression of ESCC^[10,11]. hTERT on 5p is associated with the prognosis of patients with carcinomas of the breast, lung and ESCC as reported previously^[10,12,13]. One of the potential candidate genes in the region includes JS-1 and JK-1^[13].

Deletion of chromosome 3p is one of the most frequent allelic imbalances in ESCC detected by CGH^[3,4]. In our study, loss of 3p was detected in 65% in ESCC. Possible candidate tumor suppressor genes on these region were FHIT (fragile histidine triad)^[15], catenin (CTNNB1)^[16], and von Hippel-landau gene^[17]. Our results of proximal 3p loss mainly in ESCC may indicate a specific tumor suppressor gene at this locus involved in ESCC in high-risk areas. Loss of 8p was found in 38% of ESCC cases. Deletion of 8p22-pter has been reported in patients with ESCC^[5]. Mutations of the Fasciculation and elongation protein zeta-1 (FEZ1) gene at 8p22 were found in patients with ESCC^[18], and mutations of two other genes in these regions, tumor necrosis factor-

related apoptosis-inducing ligand receptor 1 (TRAIL-R1) and receptor 2 (TRAIL-R2), were found in patients with metastatic breast carcinoma^[19]. Loss of 17p was detected in 37% of ESCC. One potentially relevant gene at 17p is TP53, whose product contributes to the control of cell proliferation and malignant transformation. Inactivation of TP53 is the most common defect found in human cancer including ESCC from Henan, China. Loss of 1p occurred in 32%. The minimal common region of deletion encompasses the most distal band 1p36-pter in ESCC. Loss of chromosome band 1p36 is frequently found in many malignancies, including gastric cardia carcinoma, colon cancer, and ESCC^[20].

In 15 pairs of ESCC and their corresponding metastatic lymph nodes, the most interesting finding in this study is the gain of 6p12-6q21 detected in seven metastatic lymph node lesions but only in two corresponding primary tumors (13% *vs* 47%; *P* = 0.05). This suggests that 6p may harbor a putative oncogene that plays an important role in the ESCC progression, especially in the lymph

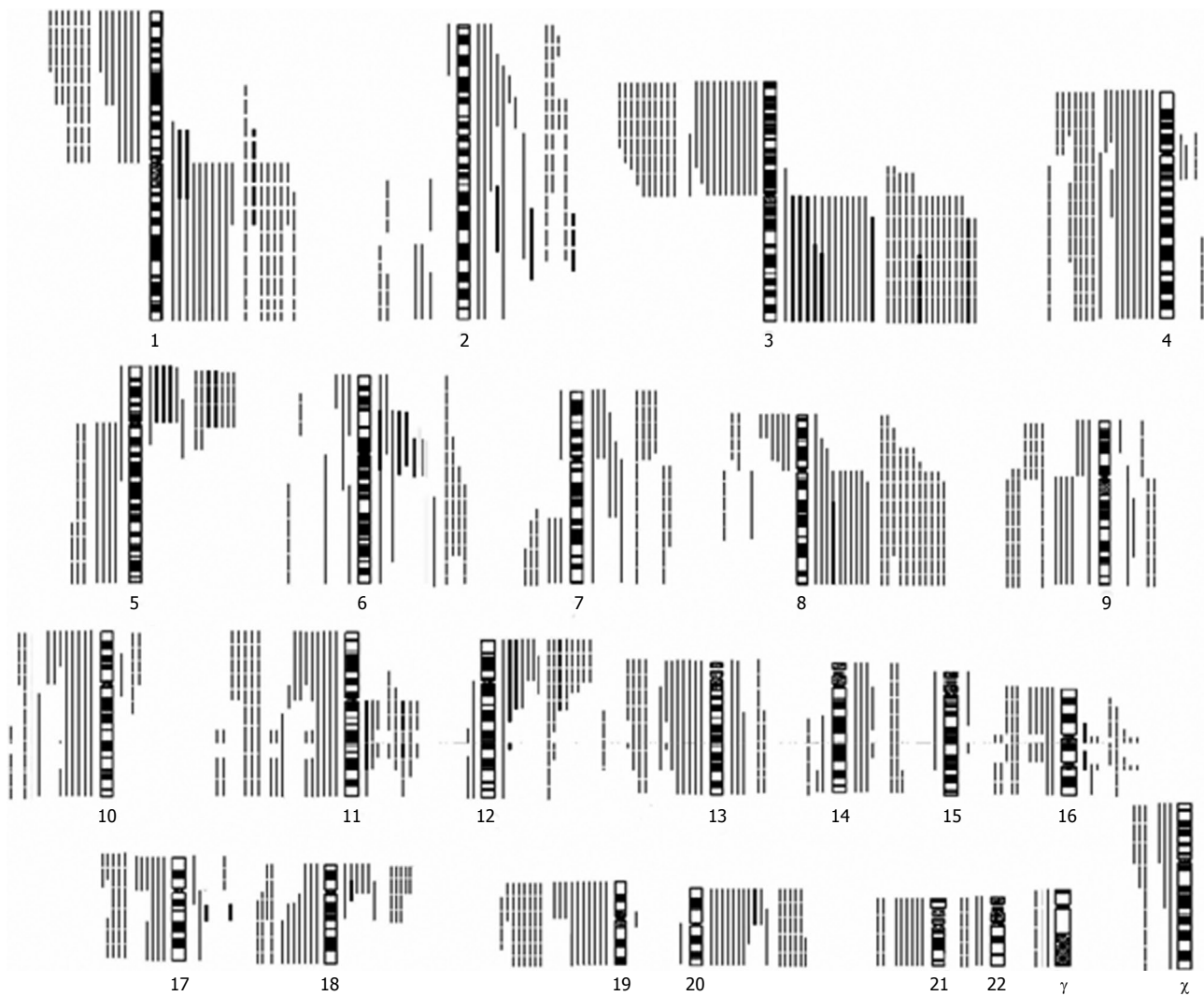


Figure 2 Copy number gains and losses in ESCC by CGH. Chromosomal localization of gains is on the right side of chromosome ideograms and that of losses are on the left side. Thick lines indicate amplified regions. Straight lines indicate lymph nodes metastasis. Conversely primary tumors are indicated by dotted lines.

node metastasis. One study shows that regions 6p12-q14 harbor the CCND3 gene, which shares 53.1% homology to CCND1. The CCND1 gene is amplified in 30% of ESCC^[21]. Regions of 6p12 are found to harbor runt-related transcription factor (RUNX) gene, which is associated with cell migration and invasion^[22]. The gain of 6p is frequently detected in CGH studies in other tumors including uveal melanoma and Barrett's adenocarcinoma^[23,24]. Most interestingly, in three cases, the high-level amplification of 6p12 was detected only in metastatic tumors. This implies that the overexpression of an oncogene(s) at 6p12 confers a selective advantage in ESCC. Moreover, this finding provides a candidate minimum amplification region at 6p12 for further studies and gene cloning. Another interesting finding in this study is the gain of 20p that was detected in 11 metastatic lymph node lesions but only in five corresponding primary tumors. The difference is significant in 20p gain between primary tumors and their metastatic lymph node lesions of ESCC (40% *vs* 73%, $P = 0.03$). This suggests that 20p may harbor an oncogene and play an important part in ESCC progression especially in lymph node metastasis. Little is known about the relationship between 20p and the lymph node metastasis

Table 3 Chromosomal aberrations with primary ESCC and lymph node metastasis

Chromosomal changes	Primary tumor (%)	Lymph node metastasis %	<i>P</i> value
Gain of 6p	2/15 (13)	7/15 (47)	0.05
Gain of 20p	5/15 (40)	11/15 (73)	0.03
Loss of 10p	2/15 (13)	8/15 (53)	0.05
Loss of 10q	2/15 (13)	7/15 (47)	0.05

of ESCC. Heselmeyer *et al* demonstrated that gains of 6p and 20p were connected with advanced-stage cervical carcinomas^[25]. Hu *et al* demonstrated the over-expression of CDC25B gene in ESCC^[26]. Possible candidate gene on 20p includes CDC25B and proliferating cell nuclear antigen (PCNA), each of which plays an important role at specific stages of cell cycle progression.

Loss of 10p was seen in eight metastatic lymph nodes but only in two corresponding primary tumors. The difference between primary tumors and their metastatic lymph node lesions of ESCC was significant (13% *vs* 53% $P = 0.05$). Loss of 10q was detected in seven metastatic lymph nodes but only in two corresponding primary

tumors. The difference between primary tumors and their metastatic lymph node lesions of ESCC was significant (13% *vs* 47% $P = 0.05$). These alterations had also been found in other carcinoma metastases, such as the head-neck squamous cell carcinoma (HNSCC)^[27] and Spitzoid malignant melanoma^[28]. A role of the 10q deletion in tumor progression is however conceivable since additional germline mutations in the PTEN gene (on chromosome 10q23) have been shown to be present in Crowden's disease^[29].

In this study, more genetic changes were found in lymph node metastasis than in their corresponding primary lesions, especially the losses (9.5/case *vs* 6.0/case). Previous studies showed that DNA copy number losses were more common in the metastases than in the primary larynx tumors and HNSCC^[30,31]. In ESCC, DNA losses seem to be more closely associated with metastases than DNA amplification.

In conclusion, using the CGH technique to detect chromosomal aberrations in both the primary tumor and its metastatic lymph nodes of ESCC, gains of 8q, 3q, 5p and losses of 3p, 8p, 9q and 13q were specifically implicated in ESCC in Linzhou population. Furthermore, gains of 6p, 20p and loss of 10pq may contribute to the lymph metastasis of ESCC. These findings suggest that the gains and losses of chromosomal regions may contain ESCC-related oncogenes and tumor suppressor genes. The gains and losses of chromosomal regions identified in this study provide important theoretic information for identifying and cloning novel ESCC-related oncogenes and tumor suppressor genes. Finally, this study provides a practicable model to detect specific genetic change related to tumor metastasis by comparing the primary tumor with its corresponding metastatic tumor using the CGH technique.

COMMENTS

Background

Esophageal squamous cell carcinoma (ESCC) is one of the leading causes of cancer-related death in Linxian, Henan Province in northern China, with a mortality rate of 161/100 000 for male and 103/100 000 for female. However, the mechanisms of human esophageal multistage carcinogenesis in this area, especially the difference in genetic changes between primary ESCC and metastatic lymph nodes are largely unknown.

Research frontiers

In this study, the authors applied comparative genomic hybridization (CGH) to ESCC to elucidate genetic aberrations in carcinogenesis and lymph node metastasis. To the knowledge of the authors, though CGH analysis of ESCC has been reported, no information is available concerning the relationship between genetic changes and the biologic characteristics of ESCC. The gains of 3q, 5p, 8q23-ter and 20q and deletions of 3p25, 4p, 6q21, 9q22.3-q31, 9p, 11q22-qter, 13q12-13, 18q22.3, and 19q had been detected as most frequent chromosomal alterations in ESCC. Our CGH results are generally consistent with other CGH studies.

Innovations and breakthroughs

The present CGH study provides the first record of chromosomal imbalances occurring in ESCC tumors and corresponding lymph nodes in Linxian, Henan Province in northern China. The major findings of this paper are that gains of 8q, 3q, 5p and losses of 3p, 8p, 9q and 13q were specifically implicated in ESCC in Linzhou population, and gains of 6p, 20p and loss of 10pq may contribute to the lymph metastasis of ESCC. These loci may harbor the genes in the development and/or progression of ESCC.

Applications

These loci provide important theoretic information for identifying and cloning novel ESCC-related oncogenes and tumor suppressor genes. Further studies are necessary to identify specific genes of these chromosomal regions and their functions. The target tumor susceptibility genes will be further characterized by mutation analysis. Single strand conformation polymorphism (SSCP) and sequencing analysis will be used to screen the mutation.

Terminology

Comparative genomic hybridization (CGH); Single strand conformation polymorphism (SSCP).

Peer review

This is a nicely written and presented paper with 37 cases of primary esophageal cancer and matched metastatic tissues. Although the number of studied cases is small, it is a useful contribution to the literature.

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VIRAL HEPATITIS

Effects of two novel nucleoside analogues on different hepatitis B virus promoters

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Abstract

AIM: To explore the effects of the nucleoside analogues β -L-D4A and β -LPA on hepatitis B virus (HBV) promoters.

METHODS: Four HBV promoters were amplified by polymerase chain reaction (PCR) and subcloned into the expression vector pEGFP-1. The four recombinants controlled by HBV promoters were confirmed by restriction analysis and sequencing. Human hepatoma HepG2 cells transfected with the recombinant plasmids were treated with various concentrations of β -L-D4A and β -LPA. Then, enhanced green fluorescent protein (EGFP)-positive cells were detected by fluorescence microscopy and using a fluorescence activated cell sorter (FACS).

RESULTS: Four HBV promoters were separately obtained and successfully cloned into pEGFP-1. Expression of EGFP under the control of the surface promoter (Sp) and the X promoter (Xp) was inhibited by β -L-D4A in a dose-dependent manner, while expression of EGFP under the control of the core promoter (Cp) and Xp was inhibited by β -LPA in a dose-dependent manner.

CONCLUSION: The two novel nucleoside analogues investigated here can inhibit the activities of HBV promoters in a dose-dependent manner. These findings may explain the mechanisms of action by which these two novel compounds inhibit HBV DNA replication.

INTRODUCTION

Hepatitis B virus (HBV) is the leading cause of chronic hepatitis in the world^[1]. According to the World Health Organization, over 350-million people (5% of the world population) are chronically infected with HBV. Although safe and effective vaccination for HBV is available in developing countries^[2-4], there is still no effective treatment for the millions of chronically infected individuals^[5]. Consequently, long-term infection with chronic HBV could lead to cirrhosis and hepatocellular carcinoma^[6,7]. In light of these facts, it is evident the discovery and development of novel antiviral agents for the treatment of HBV is an extremely important undertaking.

The number of formally approved anti-HBV drugs is limited. The necessity for new compounds acting on a variety of molecular targets within the viral replicative cycle is crucial. Thus, it remains important to discover new antiviral drugs, and to investigate new potential targets such as uncoating, transcription, packaging, excretion, or synthesis of cccDNA^[8,9].

In our previous work^[10-13], we synthesized two novel nucleoside analogues, β -L-D4A and β -LPA (Figure 1), and studied their inhibitory actions against HBV as well as their cytotoxicities. β -L-D4A and β -LPA possess potent inhibitory effects on the replication of HBV (EC_{50} = 0.2 and 0.01 μ mol/L, respectively) with little cytotoxicity (IC_{50} = 200 and 50 μ mol/L, respectively) or mitochondrial toxicity. Their TI values are 1000 and 5000, respectively. Therefore, they are expected to be developed as new clinical anti-HBV drugs. Our previous work also showed these two compounds possessed significant anti-HBV effects at the transcription level by inhibiting the

production of HBV RNA. Therefore, we supposed the two compounds might act by inhibiting the activities of HBV promoters.

Complementary DNA for the *Aequorea victoria* green fluorescent protein (GFP) produces a fluorescent product when expressed in prokaryotic or eukaryotic cells. Because exogenous substrates and cofactors are not required for this fluorescence, GFP expression can be used to monitor gene expression and protein localization in living organisms^[14-16]. pEGFP-1 is a promoterless EGFP vector, which can be used to monitor transcription from different promoters and promoter/enhancer combinations inserted into the Multiple Cloning Site located upstream of the EGFP coding sequence^[17,18]. Hence, we chose pEGFP-1 as an expression vector to monitor the activities of HBV promoters. In this study, we explored the effects of our two novel nucleoside analogues on HBV promoters to uncover the mechanism underlying their anti-HBV effects.

MATERIALS AND METHODS

Materials

β -L-D4A and β -LPA were synthesized by ourselves with the help of the Pharmaceutic College of Wuhan University, and identified by infrared, mass spectra, and nuclear-magnetic resonance. Lamivudine was provided by Professor Cheng YC (School of Medicine, Yale University, New Haven, CT, USA). These compounds were dissolved in phosphate-buffered saline (PBS). The expression vector pEGFP-1 was purchased from BD ClonTech. Plasmid p3.6 II was a kind gift from Prof. Wang Yuan (Institute of Biochemistry and Cell Biology, Shanghai Institute for Biological Sciences, Chinese Academy of Sciences). *E. coli* (Dh5 α) and human hepatoma HepG2 cells were preserved by our institute. Fetal bovine serum (FBS) and Dulbecco's modified Eagle's medium (DMEM) were purchased from Hyclone Corp. All other reagents used were of analytical grade.

Polymerase chain reaction (PCR)

The desired four fragments were HBV preS gene promoter (preSp), Sp, Cp (including enhancer II) and Xp (including enhancer I)^[19-23]. PCR was employed to amplify the four fragments from p3.6 II, a plasmid containing the HBV complete genome (adr subtype). Specific primers were designed by us and synthesized at Sangong Company (Shanghai, China). The primers used are listed in Table 1. The lower-case nucleotides indicate the recognition sites for restriction endonucleases Acc65 I and Age I (Fermentas, USA). PCR products were separated by agarose gel electrophoresis. Fragments of interest were withdrawn and directly ligated into pGEM-T vector (Promega, USA). Positive clones were then screened by virtue of a blue/white screening system and sequenced after small-scale extraction. The four positive plasmids were cleaved by Acc65 I and Age I, and fragments containing the HBV promoters were purified.

Construction of EGFP expression vectors controlled by HBV promoters

The expression vector pEGFP-1 was digested by Acc65 I

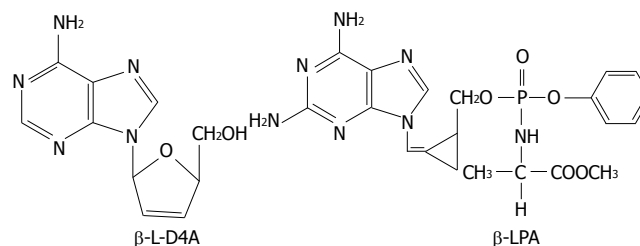


Figure 1 Structures of β -L-D4A and β -LPA.

Table 1 Primer sequences used for PCR

Target	Primer sequences	Product size (bp)
preSp	F5'-GACAAAggtaccAAACCATATTATCC-3' R5'-GAGGAccggtAACAGAAAGATTCGT-3'	229
Sp	F5'-GATTGgtaccTCAACCCCAACAAG-3' R5'-GAAAAAccggtCCTGTAACACGAG-3'	458
Cp	F5'-CCACCggtaccTGCCCAAGGTCCTTA-3' R5'-TCCAAAccggtTATACGGGTCAATG-3'	302
Xp	F5'-GTATggtaccGAATTGTGGGTCTTTTG-3' R5'-ACGTAAACAccggtCGTCCCGCGC-3'	445

and Age I and vector fragments were collected. Then, the fragments containing HBV promoters were mixed with vector fragments at a ratio of 5 to 1, and these fragments were ligated using T4 DNA ligase (Promega, USA) at 16°C overnight. The ligated products were used to transform *E. coli* (Dh5 α). Plasmids extracted from *E. coli* were analyzed by restriction enzymes and sequencing, and finally, the four promoter-controlled EGFP expression vectors pEGFP-Sp, pEGFP-preSp, pEGFP-Cp and pEGFP-Xp were produced.

Expression assays

HepG2 cells were incubated in DMEM medium with 10% (vol/vol) FBS at 37°C in a moist atmosphere containing 5% CO₂/95% air. The cells were inoculated at a density of 3×10^5 /mL per well in 24-well tissue culture plates. Twenty-four hours after plating, the four expression vectors were transfected into HepG2 cells (1 μ g DNA per well) on different plates using Lipofectamine2000 (Invitrogen), according to manufacturer's instructions (one well was left as a negative control group, which was needed as reference for FACS). After a 6 h incubation, the transfected cells were treated with various concentrations of β -L-D4A (0.08 μ mol/L in 4 wells, 0.4 μ mol/L in 4 wells, 2 μ mol/L in 4 wells, 10 μ mol/L in 4 wells). Four wells of cells were treated with lamivudine at 1 μ mol/L as a comparative group, and 3 wells were not treated with any drug as a positive control group (that is, a blank control). The cells were grown in the presence of drugs for 42 h. EGFP-positive cells were detected using an inverted fluorescence microscope (ZEISS, Axiovert40) with an excitation wavelength of 488 nm. Then, all cells were digested with 0.25% trypsin; digestion was terminated by FBS and the cells were re-suspended in 500 μ L of PBS per well before being subjected to flow cytometry (Becton Dickinson) analysis. The same experiments were performed with β -LPA.

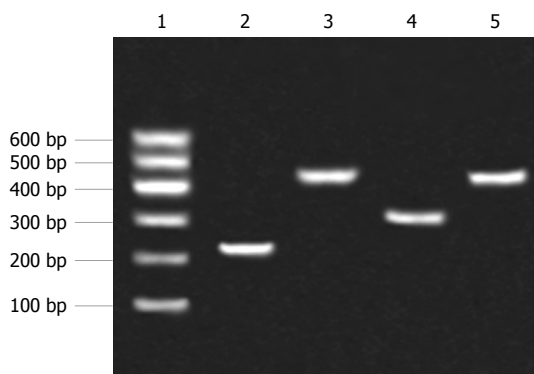


Figure 2 The HBV promoters obtained from PCR were separated by agarose gel electrophoresis. Lane 1: Marker; Lane 2: preSp; Lane 3: Sp; Lane 4: Cp; Lane 5: Xp.

Statistical analysis

Data are expressed as mean \pm SD. Each experiment was repeated at least three times. Differences were considered statistically significant when $P < 0.05$, as analyzed by one way analysis of variance and Tukey's post-hoc test. The analysis was conducted using the SPSS12.0.

RESULTS

Validation of cloning

After ligation of promoter fragments into pGEM-T vector, PCR products (Figure 2) were sequenced by Bioasia (Shanghai, China). Using BLAST searches of Entrez (NCBI), the sequencing results were proved to be consistent with the template (p3.6 II). The four recombinants pEGFP-Sp, pEGFP-preSp, pEGFP-Cp and pEGFP-Xp were identified by restriction enzymes (Figure 3).

Detection of EGFP-positive cells

EGFP-positive cells could be seen by fluorescence microscopy among HepG2 cells transfected with pEGFP-Sp, pEGFP-Cp and pEGFP-Xp, but few could be seen among HepG2 cells transfected with pEGFP-preSp (Figure 4). These findings suggested HBV promoters could control the expression of EGFP.

FACS analysis

The percentage of EGFP-positive cells in each well was obtained by FACS. β -L-D4A inhibited the expressions of EGFP under the control of Sp (Figure 5, one representative picture was chosen from each group) and Xp in a dose-dependent manner, but had no effect on the expression of EGFP under the control of preSp and Cp; by contrast, β -LPA inhibited the expression of EGFP under the control of Cp and Xp in a dose-dependent manner, but had no effect on the expression of EGFP under the control of preSp and Sp; lamivudine could not inhibit the expression of EGFP under the control of any HBV promoter. The results are summarized in Tables 2 and 3.

DISCUSSION

HBV infection remains a major public health problem

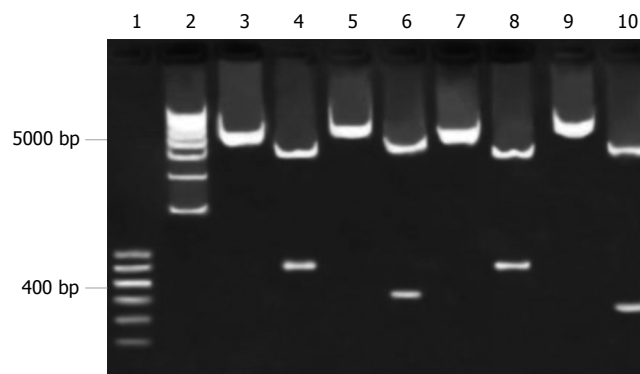


Figure 3 Restriction analysis of recombinant vectors. Lanes 1, 2: Marker; Lanes 3, 4: pEGFP-Sp before and after being digested by Acc65 I and Age I; Lanes 5, 6: pEGFP-Cp before and after being digested by Acc65 I and Age I; Lanes 7, 8: pEGFP-Xp before and after being digested by Acc65 I and Age I; Lanes 9, 10: pEGFP-preSp before and after being digested by Acc65 I and Age I.

worldwide. Antiviral treatment of chronic Hepatitis B currently relies on immune modulators such as interferon alpha and its pegylated form, and viral polymerase inhibitors which belong to the nucleoside and nucleotide analog family^[24]. Unfortunately, interferon alpha therapy is associated with several side effects, and the response rate for those receiving treatment has been unsatisfactory^[25,26]. Because of the slow kinetics of viral clearance and spontaneous viral genome variability, viral mutants resistant to nucleoside analogs may be selected^[27,28]. Thus, drugs targeting other unique viral targets are needed.

Our results indicate β -L-D4A and β -LPA can inhibit the activities of HBV promoters in a dose-dependent manner. On the other hand, expression of EGFP was not inhibited by lamivudine. This shows lamivudine has no effect on HBV promoters and suggests the anti-HBV effects of the two novel nucleoside analogues are mediated by mechanisms different from those used by lamivudine. HBV, a causative agent of hepatitis and hepatocellular carcinoma, contains a 3.2-kb partially double-stranded DNA genome. Upon infection of a host, the viral genome is transcribed to generate a 3.5-kb pregenomic RNA used as a template for viral replication. The pregenomic/core promoter is responsible for the synthesis of this 3.5-kb pregenomic RNA; therefore, regulation of this promoter is important in the viral life cycle^[29]. The 3.5-kb RNA also serves as a template for the synthesis of polymerase and nucleocapsid core protein. In addition to the 3.5-kb RNA, three more transcripts are generated from the HBV genome. The large surface antigen is synthesized from a 2.4-kb RNA, and the major and middle antigens are synthesized from 2.1-kb transcripts. The X-gene product (HBx) is synthesized from the smallest 0.8-kb RNA^[29]. The transcriptions of these RNAs are governed by the pre-S, surface, and X promoters, respectively^[30,31]. Thus, HBV promoters are crucial for HBV transcription and play an important role in the HBV replicative cycle. Our previous study shows that β -L-D4A and β -LPA possess potent inhibitory effects on the replication of HBV *in vitro* with little cytotoxicity or mitochondrial toxicity, and can inhibit the expression of HBV antigens at high concentrations^[10,13]; these findings can be explained by a model in which the two compounds inhibit

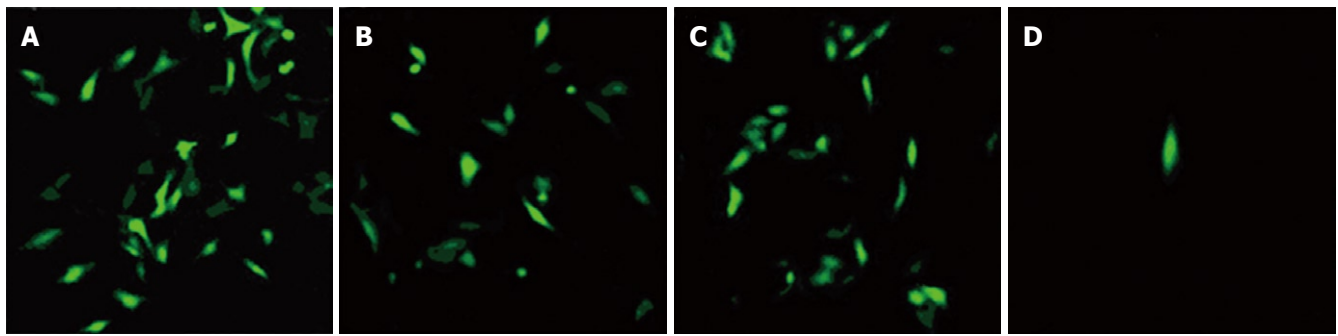


Figure 4 Forty-eight hours after transfection, EGFP positive cells were detected by fluorescence microscopy ($\times 100$). **A:** HepG2 cells transfected with pEGFP-Sp; **B:** HepG2 cells transfected with pEGFP-Cp; **C:** HepG2 cells transfected with pEGFP-Xp; **D:** HepG2 cells transfected with pEGFP-preSp.

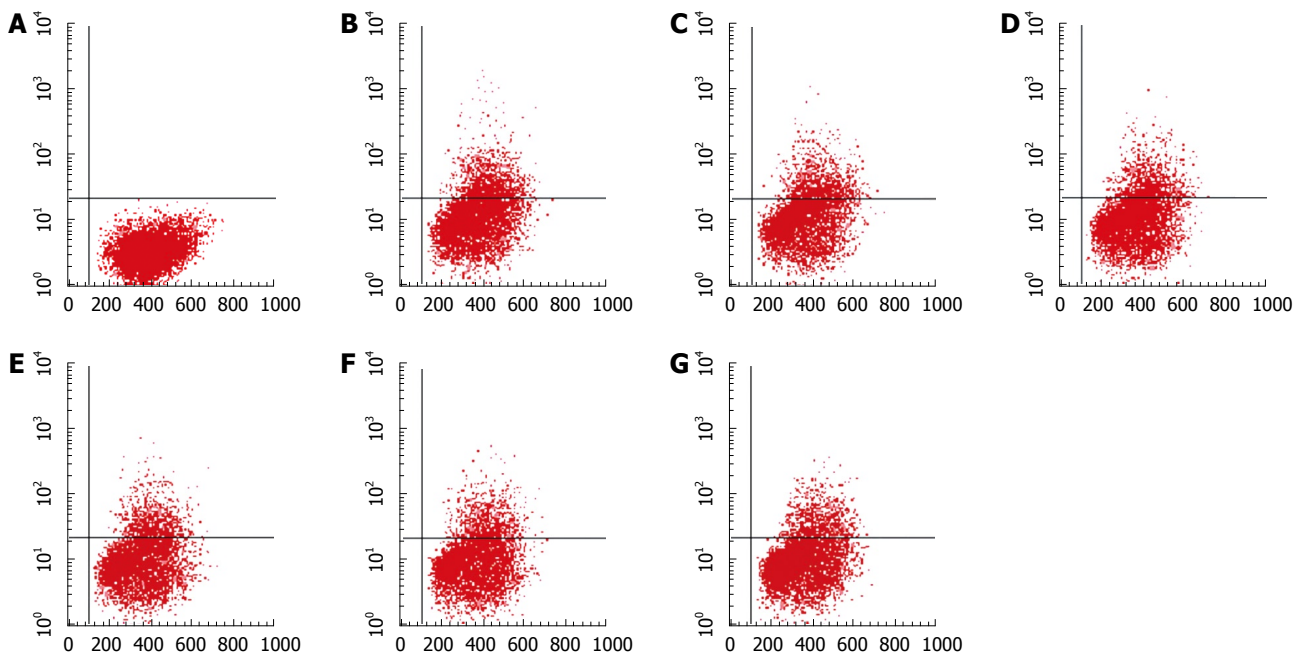


Figure 5 β -L-D4A inhibited the expression of EGFP in HepG2 cells transfected with pEGFP-Sp, as determined by FACS analysis. **A:** HepG2 cells not transfected with pEGFP-Sp, not treated with drug, the percentage of EGFP-positive cells was 0; **B:** HepG2 cells transfected with pEGFP-Sp, not treated with drug, the percentage was 21.42%; **C:** HepG2 cells transfected with pEGFP-Sp, treated with Lamivudine at 1 μ mol/L, the percentage was 21.14%; **D-G:** HepG2 cells transfected with pEGFP-Sp, treated with β -L-D4A at various concentrations (0.08, 0.4, 2, 10 μ mol/L), the percentages were 18.76%, 17.31%, 15.53%, 13.65% respectively.

Table 2 Effect-dosage relationship of the inhibition of the expression of EGFP under the control of the Sp and Xp promoters by β -L-D4A

Dosage (μ mol/L)	n	EGFP-positive cells (%) (mean \pm SD)		Inhibition rate (%)	
		Sp	Xp	Sp	Xp
Control	3	21.26 \pm 0.25	18.52 \pm 1.25	0.0	0.0
(Lamivudine)	4	21.00 \pm 0.43	18.37 \pm 1.01	1.2	0.8
0.08	4	18.76 \pm 0.41 ^b	16.47 \pm 0.49 ^b	11.8	11.1
0.4	4	17.31 \pm 0.41 ^b	15.68 \pm 0.36 ^b	18.6	15.3
2	4	15.54 \pm 0.48 ^b	13.54 \pm 0.59 ^b	26.9	26.9
10	4	11.33 \pm 0.32 ^b	10.84 \pm 0.81 ^b	46.7	41.5

^b $P < 0.01$ vs Blank control. EGFP: Enhanced green fluorescent protein.

Table 3 Effect-dosage relationship of the inhibition of the expression of EGFP under the control of the Cp and Xp promoters by β -LPA

Dosage (μ mol/L)	n	EGFP-positive cells (%) (mean \pm SD)		Inhibition rate (%)	
		Cp	Xp	Cp	Xp
Control	3	13.99 \pm 0.29	18.58 \pm 0.39	0.0	0.0
(Lamivudine)	4	13.80 \pm 0.63	18.46 \pm 0.49	1.3	0.6
0.002	4	12.98 \pm 0.16 ^b	17.54 \pm 0.31 ^b	7.2	5.6
0.01	4	11.96 \pm 0.75 ^b	16.18 \pm 0.21 ^b	14.5	12.9
0.05	4	10.88 \pm 0.43 ^b	14.63 \pm 0.37 ^b	22.2	21.3
1	4	8.79 \pm 0.56 ^b	11.94 \pm 1.37 ^b	37.7	35.7

^b $P < 0.01$ vs Blank control. EGFP: Enhanced green fluorescent protein.

the activities of HBV promoters, as shown in the present study. Thus, HBV promoters may be molecular targets of these two compounds. To confirm this, DNase I footprint-

ing assays should be performed in the future. Two main points are worthy of mention here. First, although HBV promoters are crucial to the HBV life cycle, no research on

anti-HBV drugs using promoters as molecular targets has been reported to date. Therefore, our effort to explore the effects of these two novel nucleoside analogues on HBV promoters is valuable and necessary. Second, four EGFP expression vectors containing different HBV promoters were successfully constructed by us. These vectors offer us the ability to monitor the activities of HBV promoters and provide an effective way to detect the effects of novel anti-HBV drugs on HBV promoters. Compared with the chloramphenicol acetyltransferase (CAT) reporter gene, the EGFP reporter gene has more advantages. Analysis of EGFP expression easier and there is no pollution from radiation. In summary, we have shown that β -L-D4A can inhibit the activities of Sp and Xp promoters, and that β -LPA can inhibit the activities of Cp and Xp promoters, in dose-dependent manners. These findings may help us to explain the mechanisms of action of these two novel compounds.

COMMENTS

Background

Hepatitis B virus (HBV) infection remains a global health problem. Currently, antiviral treatment of chronic Hepatitis B relies on interferon alpha and nucleoside analogs that inhibit viral polymerase. However, interferon alpha therapy has many side effects, while the use of nucleoside analogs can lead to the emergence of resistant viral mutants. Thus, development of novel antiviral agents against HBV is an extremely important undertaking.

Research frontiers

All of the approved chemotherapeutic drugs for the treatment of HBV hepatitis are nucleoside analogs targeting HBV DNA polymerase. Drugs targeting other unique viral targets are needed.

Innovations and breakthroughs

Although HBV promoters are crucial to HBV's life cycle, research on anti-HBV drugs targeting HBV promoters has not yet been reported. Therefore, our efforts to explore the effects of two novel nucleoside analogues on HBV promoters are valuable and necessary.

Applications

This work may help to explain the mechanisms underlying the anti-HBV actions of β -L-D4A and β -LPA, which possess potent inhibitory effects on the replication of HBV, with little cytotoxicity or mitochondrial toxicity. Therefore, they are expected to be developed as new clinical anti-HBV drugs.

Terminology

Green fluorescent protein (GFP) was firstly used as a marker of gene expression by Chalfie (*Science* 1994; 263: 802-805), and later developed as an EGFP reporter gene, which uses GFP to monitor gene expression and protein localization in living organisms.

Peer review

The authors explored nucleoside analogues β -L-D4A and β -LPA's effects on HBV promoters.

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BASIC RESEARCH

Immune-mediated anti-neoplastic effect of intratumoral RSV envelope glycoprotein expression is related to apoptotic death of tumor cells but not to the size of syncytia

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colon cancer cell lines with Ad.RSV-F or Ad.RSV-F/G caused apoptotic cell death, in contrast to transduction with Ad.RSV-G or Ad.RSV-F_{sol}, suggesting an importance of the mode of cell death. Overall, these findings provide insight into improved tumor vaccination strategies relying on the intratumoral expression of viral fusogenic membrane proteins.

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Key words: Adenoviral vectors; Tumor vaccination; Fusogenic membrane protein; Colorectal cancer; Syngeneic tumor model

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Abstract

AIM: To promote the development of improved tumor vaccination strategies relying on the intratumoral expression of viral fusogenic membrane proteins, we elucidated whether the size of syncytia or the way tumor cells die has an effect on the therapeutic outcome.

METHODS: In two syngeneic subcutaneous murine colon cancer models we assessed the anti-neoplastic effect on vector-treated and contralateral untreated tumors.

RESULTS: Intratumoral injection of a replication-defective adenovirus encoding respiratory syncytial virus fusion protein (RSV-F) alone (Ad.RSV-F) or together with the attachment glycoprotein RSV-G (Ad.RSV-F/G) led to a significant growth reduction of the vector-treated and contralateral untreated tumors. The treatment response was associated with a strong tumor-specific CTL response and significantly improved survival with medians of 46 d and 44 d, respectively. Intratumoral injection of Ad.RSV-G or a soluble RSV-F encoding adenovirus (Ad.RSV-F_{sol}) had no significant anti-neoplastic effect. The median survival of these treatment groups and of Ad.Null-treated control animals was about 30 d.

CONCLUSION: Although *in vitro* transduction of colon cancer cell lines with Ad.RSV-F/G resulted in about 8-fold larger syncytia than with Ad.RSV-F, the *in vivo* outcome was not significantly different. Transduction of murine

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INTRODUCTION

For a potent and long-lasting tumor therapy it is desirable to eradicate the primary tumor and also to induce an anti-tumor immunity to prevent the spread and recurrence of tumor cells. Recent advances in tumor immunology have identified various tumor-associated antigens, and this has facilitated the development of vaccine strategies for cancer. However, the use of tumor-associated antigens as a vaccine component is limited to cancer patients with a known tumor antigen^[1]. To circumvent this limitation, some cancer vaccination strategies use killed tumor cells or lysates delivered in combination with adjuvants or cytokines, which probably include both known and unknown antigens^[2,3]. Furthermore, gene transfer of cytokines, MHC molecules, costimulatory molecules or tumor antigens to tumor cells has been used to enhance the visibility of tumor cells to immune effector cells^[4,5].

In 1994, Polly Matzinger presented a new theory called the danger model, suggesting a specific immune

response develops as a result of danger detection rather than discrimination between self and non-self antigens^[6,7]. According to the danger model, the immune surveillance system fails to detect tumor antigens because transformed cells do not send any danger signals^[7]. Danger signals are thought to act by stimulating dendritic cells to mature so that they can present foreign antigens and stimulate T cells^[8-11]. During infections, microbial components provide signals that alert the immune system to danger and promote the generation of immunity^[8,12]. Dying mammalian cells have also been found to release danger signals^[13-16]. In the absence of such signals there is often no immune response or tolerance may develop.

Linardakis *et al* demonstrated in a syngeneic murine B16 melanoma model that the expression of fusogenic membrane protein G from vesicular stomatitis virus (VSV-G) can enhance the efficacy of a weak allogeneic vaccine^[17]. Fusogenic membrane glycoproteins were introduced as a new class of therapeutic genes for cancer gene therapy by Bateman *et al*, who demonstrated that expression of these proteins alone resulted in a significantly greater tumor growth control than suicide prodrug systems^[18]. The fusion of viral envelopes with cellular membranes is an essential step mediating the entry of enveloped viruses into host cells. This process is mediated by specific viral proteins such as the fusion (F) protein of paramyxoviruses^[19-21]. A member of this virus family, human respiratory syncytial virus (RSV), encodes three envelope glycoproteins, namely the major attachment glycoprotein (G)^[22], the small hydrophobic (SH) protein, which blocks TNF- α mediated apoptosis^[23], and the fusion glycoprotein (F), which mediates virus-cell and cell-cell fusion^[24], creating the characteristic syncytia for which the virus is named.

Previously, we demonstrated in a syngeneic bilateral subcutaneous MC38 and Colon26 colon cancer model in immunocompetent mice that the injection of one tumor with a replication-defective adenovirus encoding RSV-F resulted not only in tumor growth reduction of the treated tumor, but also of the second, untreated contralateral tumor^[25]. We observed qualitatively similar effects with fusogenic membrane proteins of measles virus (MV-H/F)^[26]. The effects were associated with a tumor-specific cytotoxic T cell (CTL) response and a pronounced infiltration of tumors with natural killer cells and macrophages.

In an attempt to promote the development of improved tumor vaccination strategies that rely on intratumoral expression of viral fusogenic membrane proteins, in this study we elucidated factors that might influence the induction of a systemic anti-tumor response. Using the same bilateral subcutaneous tumor models we demonstrated that treatment of one cutaneous tumor with a replication-defective adenovirus encoding RSV-F alone (Ad.RSV-F) or in combination with RSV-G (Ad.RSV-F/G) resulted in improved survival and the induction of a systemic anti-tumor immune response. Although *in vitro* transduction of tumor cells with Ad.RSV-F/G resulted in significantly larger syncytia compared with transduction of tumor cells with Ad.RSV-F, the *in vivo* treatment

outcome was not significantly influenced by the size of cell-cell fusion. Treatment of animals with an adenovirus encoding a soluble, non-fusogenic form of RSV-F (Ad.RSV-F_{sol}) or RSV-G (Ad.RSV-G) had no anti-neoplastic effect, indicating that the anti-neoplastic effects are not primarily mediated by intrinsic immunological properties of RSV envelope glycoproteins. In both tumor models we observed the induction of a systemic anti-tumor response only when RSV glycoprotein expression in the tumor cells caused syncytium formation and apoptosis, independent of the size of syncytia.

MATERIALS AND METHODS

Cells and cell culture

The murine colon adenocarcinoma cell line MC38 was obtained from Steven A. Rosenberg, NCI, NIH, Bethesda, MD. The murine adenocarcinoma cell line Colon26 was purchased from CLS (Heidelberg, Germany). The human embryonic kidney cell line 293 was obtained from Microbix Biosystems, Inc. (Toronto, Canada). The T-REx-293 cells, which stably express a tetracycline-dependent repressor, were purchased from Invitrogen (San Diego, CA, USA). Cell lines were propagated in Dulbecco's modified Eagle medium with high glucose (Invitrogen, Karlsruhe, Germany), supplemented with 10% heat-inactivated fetal bovine serum and 50 μ g/mL gentamicin. All cell lines were routinely tested for *Mycoplasma* and found to be free of contamination.

Viruses

The replication-defective Ad5-based vector Ad.GFP, which encodes enhanced green fluorescent protein (GFP) driven by the CMV-IE promoter, has been described previously^[27]. As a vector control we used Ad.Null, a replication-defective adenovirus generated with the Ad-Easy-1 system^[28] that does not encode a transgene.

The adenovirus vector Ad.RSV-F, which carries a codon-optimized cDNA for native RSV-F^[29], has been described previously^[25]. The vector Ad.RSV-F_{sol} encodes a non-fusogenic soluble form of RSV-F, which lacks the transmembrane domain and cytoplasmic tail (Δ 524-574). The vector Ad.RSV-G encodes the RSV (ATCC VR26) major attachment protein G, which was codon optimized for expression in human cells (GeneArt, Regensburg, Germany). In all adenovirus vectors, the RSV envelope glycoproteins were under the transcriptional control of the TetO2 promoter^[30]. The vector Ad.RSV-F/G encodes the RSV-F and RSV-G proteins under the transcriptional control of a bi-directional TetO2 promoter. All RSV glycoprotein encoding adenovirus vectors were generated using the Ad-Easy-1 system^[28], and are Ad5-based and E1-, E3-deleted. Infectious vectors were rescued using T-REx-293 cells in the absence of tetracycline.

All vectors were purified using the Vivapure AdenoPACK 100 kit (Vivascience, Hannover, Germany). Vector particle concentration was determined by spectrophotometry as described previously^[31] and expressed as viral particles (VP)/mL. The particle-to-PFU ratios of all vector preparations were about 30:1.

Analysis of cell-cell fusion

About 95%-100% confluent MC38 or Colon26 cell monolayers were transduced with equal amounts of Ad.GFP to enhance syncytium visibility and Ad.Null, Ad.RSV-F, Ad.RSV-F/G, Ad.RSV-G, or Ad.RSV-F_{sol}, at a multiplicity of infection (MOI) of 1000 VP/cell. Forty-eight hours after vector transduction, cells were analyzed by inverse fluorescence microscopy. Digital images were captured using a high-resolution still camera (Olympus DP50, Tokyo, Japan) attached to a fluorescence microscope (Olympus BX51, Tokyo).

Analysis of apoptosis

Fourteen hours after treatment, the annexin V binding and caspase-3/7 activity of MC38 and Colon26 cells was analyzed using the Annexin V-FITC Apoptosis Detection Kit I (BD Biosciences, San Jose, CA) or Caspase-Glo™ 3/7 assay (Promega, USA), respectively, as described previously^[32]. Changes in mitochondrial membrane potential ($\Delta\Psi_m$) were analyzed by flow cytometry using the BD MitoScreen Kit (BD Biosciences), as described previously^[32].

In vivo studies

The Animal Care and Use Committee of the Ruhr-University Bochum approved all described studies. Six- to eight-week-old female C57BL/6 and BALB/c mice were obtained from Janvier (Le Genest-St-Isle, France). To generate the syngeneic bilateral subcutaneous syngeneic tumor model, C57BL/6 or BALB/c mice received subcutaneous injections of 1×10^5 MC38 or Colon26 cells, respectively, in 100 μ L into the right hind flank and 1×10^4 cells in 100 μ L into the left hind flank. Animals were randomly assigned to treatment groups ($n = 5$ for each tumor model) when the tumor on the right hind flank reached a volume of about 200 mm³ and the tumor on the left side was palpable. On day 0 and 2, animals received 6×10^9 VP of Ad.Null, Ad.RSV-F, Ad.RSV-F/G, Ad.RSV-G, or Ad.RSV-F_{sol} in 100 μ L of PBS into the tumor on the right flank. Tumor growth was monitored at least once a week, minimum and maximum perpendicular tumor axes were measured using vernier calipers, and tumor volume was calculated using the simplified formula of a rotational ellipse ($l \times w^2 \times 0.5$). The skin thickness of 0.4 mm was subtracted from the measurements. To generate effector cells, mice were sacrificed and spleens were harvested and weighed 28 d after virus inoculation. When animals seemed to be in distress or the tumor weight exceeded 10% of the body weight, animals were euthanized by CO₂ asphyxia.

CTL assay

We analyzed the CTL response to tumor cells, using the lactate dehydrogenase (LDH)-based CytoTox 96 (Promega) assay according to the manufacturer's instructions. In brief, target cells (MC38 or Colon26) were plated at a density of 5×10^3 cells per well in round-bottomed 96-well plates. Target cells were then mixed with effector cells at the indicated ratios and co-incubated for 4 h. LDH release was determined measuring absorbance at 490 nm with a plate reader, and the specific lysis was calculated from triplicate samples as follows:

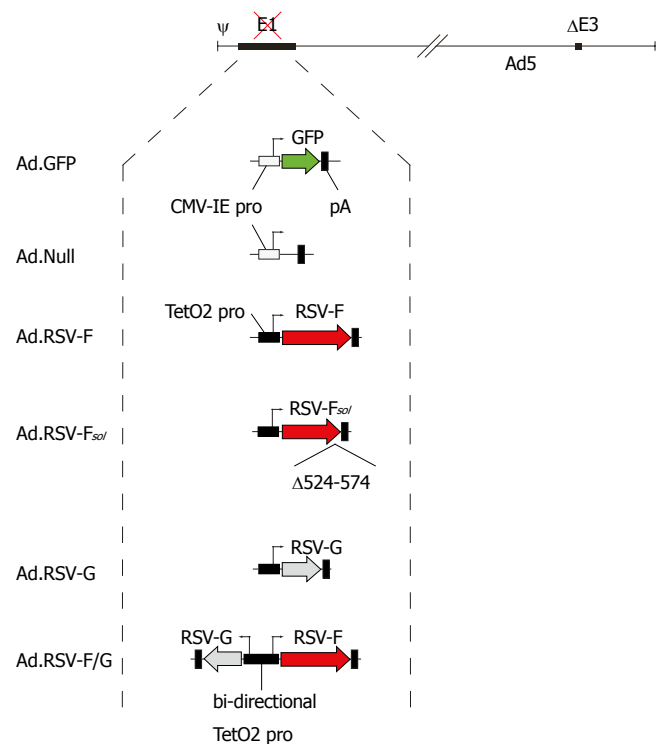


Figure 1 Adenovirus vector design. All adenovirus vectors used in this study are E1- and E3-deleted and Ad5-based. The vector Ad.GFP encodes green fluorescent protein and the vector Ad.Null served as an adenovirus vector control. The RSV glycoprotein encoding vectors carried the transgene under the transcriptional control of the doxycycline-repressible TetO2 promoter. The vector RSV-F_{sol} encodes a soluble form of the RSV fusion protein F without the transmembrane domain and cytoplasmic tail ($\Delta 524-574$).

$$\text{Specific lysis (\%)} = \frac{\text{Experimental } A_{490} - \text{Effector spontaneous } A_{490} - \text{Target spontaneous } A_{490}}{\text{Target maximum } A_{490} - \text{Target spontaneous } A_{490}} \times 100.$$

Statistical analysis

The statistical software package SPSS 15 (SPSS Inc., Chicago, USA) was used for data analysis. For comparative analysis of survival rates across treatment groups, Kaplan-Meier analysis with the log-rank test was used. Tumor volumes and spleen weights were analyzed by one-way analysis of variance (ANOVA) followed by the Tukey's honestly significantly different (HSD) test. The sizes of syncytia were determined with the image analysis software ImageJ 1.38 (<http://rsb.info.nih.gov/ij>). The calculated areas of 30 syncytia in each treatment group were compared using the Mann-Whitney Rank Sum Test.

RESULTS

Intratumoral expression of RSV-F alone or in combination with RSV-G induces an antineoplastic effect on the treated tumor as well as on the untreated contralateral tumor

In the subcutaneous syngeneic bilateral MC38 and Colon26 colon cancer model we analyzed in more detail factors that are necessary to induce a systemic anti-tumor response by intratumoral expression of RSV envelope glycoproteins encoded by replication-defective adenovirus vectors (Figure 1). To analyze whether enhanced syncytium formation improves the tumor vaccination effect, animals were treated with Ad.RSV-F or an adenovirus encoding

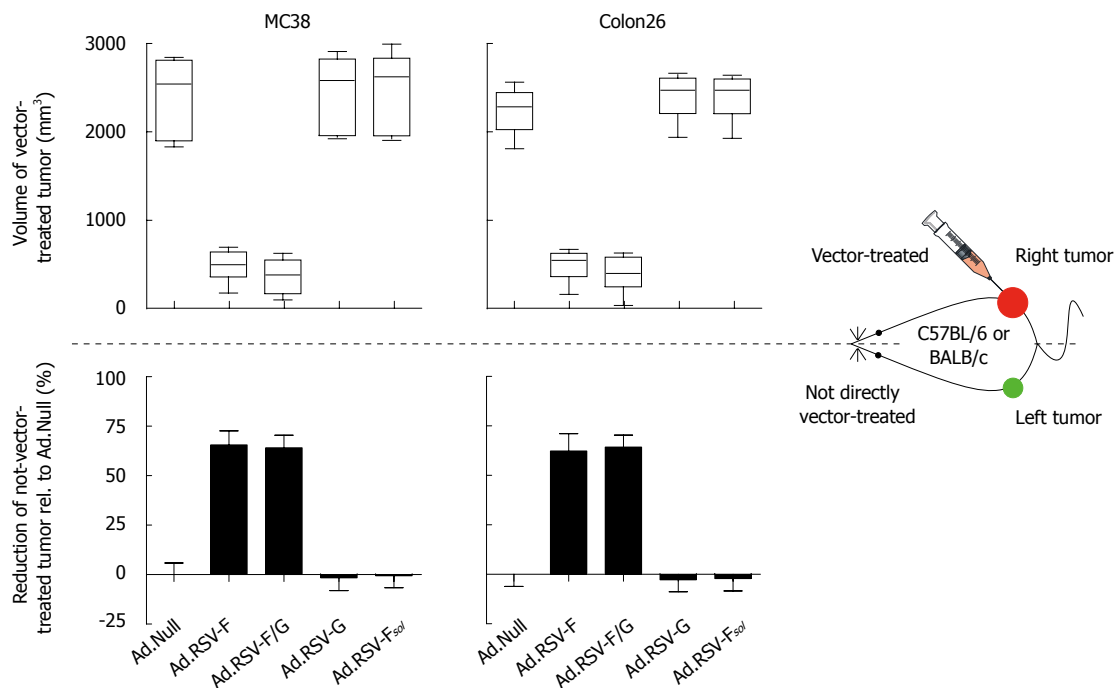


Figure 2 Intratumoral expression of RSV-F or RSV-F/G induces an anti-neoplastic effect. In a bilateral subcutaneous syngeneic MC38 or Colon26 colon cancer model, the indicated adenovirus vectors were inoculated on d 0 and d 2 into the tumor on the right flank. No viral vectors were inoculated into the tumor on the left flank. **A:** The volume of the tumor on the right flank was measured at d 28 and presented as box-and-whisker plots, showing minimum, 25th percentile, median, 75th percentile, and maximum tumor volume; **B:** The volume of the tumor on the left flank, which did not receive direct viral vector injections, was measured at d 28 and the volume reduction relative to Ad.Null-treated control animals is presented as bar graphs (mean \pm SD).

RSV-F as well as RSV-G (Ad.RSV-F/G). Furthermore, we elucidated whether syncytium formation is necessary to induce a systemic anti-tumor response or whether this effect is mediated by intrinsic immunological properties of RSV envelope glycoproteins. Animals were treated with an adenovirus encoding a soluble, non-fusogenic RSV-F without the transmembrane domain and cytoplasmic tail (Ad.RSV-F_{sol}). Treatment of animals with Ad.RSV-G served as a control. In addition we analyzed whether the tumor cells needed to undergo apoptosis to induce a systemic anti-tumor response.

As shown in Figure 2, injection of Ad.RSV-F or Ad.RSV-F/G into the right tumor resulted in about 80% and 87% reduction, respectively, in the size of vector-treated tumors at day 28 ($P < 0.001$). Importantly, treatment of the right tumor with Ad.RSV-F or Ad.RSV-F/G also resulted in about 60% reduction of the left, untreated tumor compared with Ad.Null-treated animals ($P < 0.001$). By contrast, treatment with Ad.Null, Ad.RSV-G or Ad.RSV-F_{sol} had no significant anti-neoplastic effect on either vector-treated or contralateral tumors ($P > 0.05$).

To assess whether these results are unique to MC38 cells and C57BL/6 mice (H-2^b), we repeated the syngeneic bilateral tumor model under identical conditions with Colon26 cells in BALB/c mice (H-2^d), which have contrasting susceptibilities to certain intracellular pathogens^[33,34]. As shown in Figure 2, the results were qualitatively similar to that obtained with MC38 cells.

Intratumoral expression of RSV-F alone or in combination with RSV-G results in enhanced survival

Next, we analyzed in the syngeneic bilateral MC38

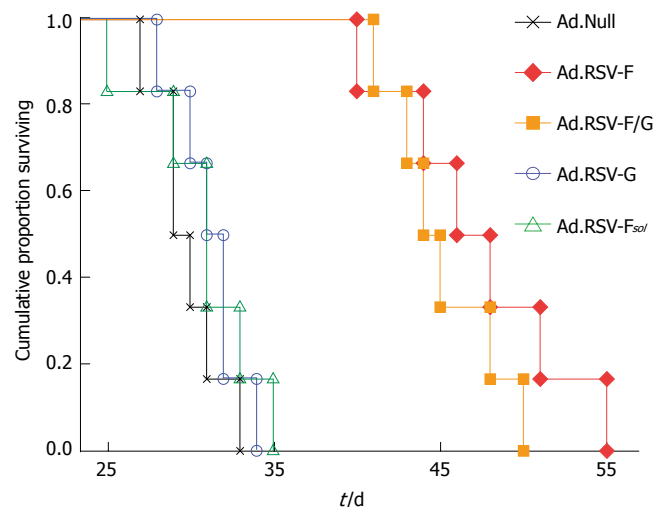


Figure 3 Kaplan-Meier survival analysis. In the bilateral subcutaneous syngeneic MC38 colon cancer model described in Figure 2, one tumor was treated with the indicated adenoviral vectors, and survival time was monitored up to d 55.

subcutaneous colon cancer model whether intratumoral expression of RSV envelope glycoproteins also results in improved survival. Kaplan-Meier survival analysis revealed that animals treated with Ad.Null had a median survival of 29 d (Figure 3). Animals that received intratumoral injections of Ad.RSV-G or Ad.RSV-F_{sol} had a median survival of 31 d ($P > 0.05$). Treatment of mice with Ad.RSV-F or Ad.RSV-F/G resulted in a significantly improved outcome with median survivals of 46 d and 44 d, respectively ($P < 0.001$).

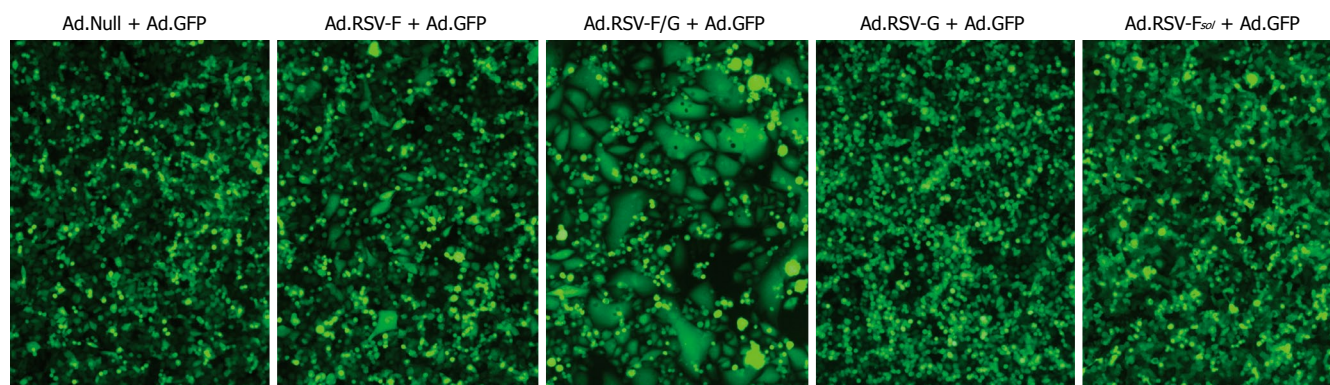


Figure 4 Fluorescence micrographs. MC38 cells were transduced *in vitro* with indicated RSV glycoprotein encoding adenoviruses and an adenovirus encoding GFP. Transduction with Ad.GFP enhanced the visibility of syncytia. Representative pictures 48 h after transduction are shown ($\times 200$). Similar data were obtained with the Colon26 cells (data not shown).

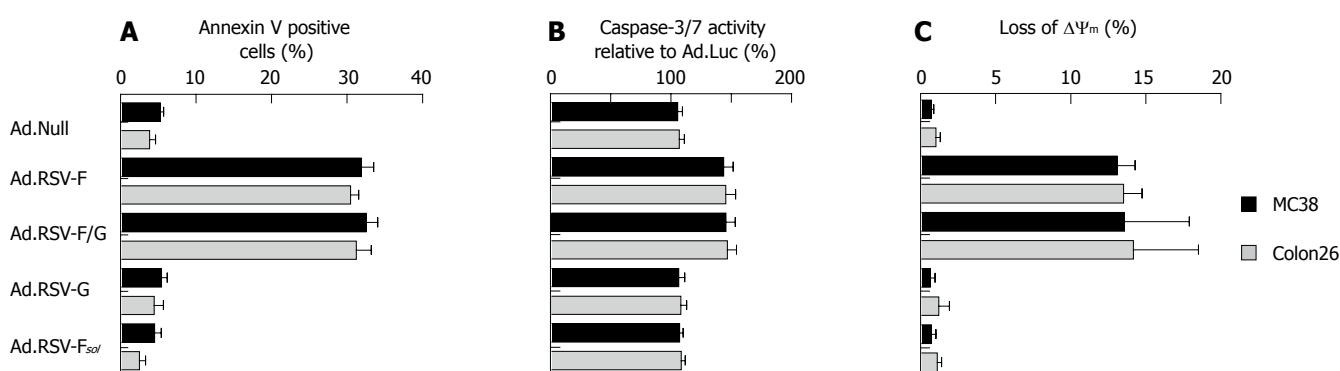


Figure 5 Analysis of apoptosis. **A:** MC38 or Colon26 cells were transduced *in vitro* with indicated adenovirus vectors and early apoptotic events were analyzed by flow cytometric measurements of phosphatidylserine translocation to the outer membrane by annexin V binding; **B:** Because apoptosis is essentially executed by proteases of the caspase family, we analyzed caspase-3/7 activity. Relative caspase activities are given as means \pm SD of three independent experiments; **C:** To measure mitochondrial alterations we determined the mitochondrial membrane potential $\Delta\Psi_m$. Ad.Null served as a control.

Expression of RSV-F alone or in combination with RSV-G results in syncytium formation in MC38 and Colon26 cells

Next, we determined whether the size of syncytium formation correlates with the treatment outcome. As shown in Figure 4, no cell-cell fusion was detectable in MC38 cells transduced with Ad.Null, Ad.RSV-G or Ad.RSV-F_{sol}. The median syncytia area of monolayers transduced under identical conditions with Ad.RSV-F or Ad.RSV-F/G was about 3 times and 25 times larger than the median area of single MC38 cells transduced with Ad.Null. Transduction of cell monolayers with Ad.RSV-F/G resulted in significantly larger syncytia than Ad.RSV-F ($P = 0.001$). Similar data were obtained with Colon26 cells (data not shown).

Expression of RSV-F alone or in combination with RSV-G results in apoptosis

To examine whether the cells need to undergo apoptosis after expression of RSV envelope glycoproteins to induce a systemic anti-tumor response, we analyzed early and late events of programmed cell death. We first analyzed the binding of annexin V-FITC to externalized phosphatidylserine, which reflects reversible membrane damage, as a marker for the early stages of apoptosis in combination with vital staining^[35]. As shown in Figure 5A,

there was no significant annexin V binding to MC38 or Colon26 cells transduced with Ad.Null. Fourteen hours after transduction with Ad.RSV-G or Ad.RSV-F_{sol}, the median percentage of annexin V-positive cells was about 6%. Transduction of the tumor cell lines with Ad.RSV-F or Ad.RSV-F/G resulted in about 32% annexin V-positive cells.

Induction of apoptosis is essentially executed by the activation of caspases in response to extrinsic or intrinsic stimuli. Activation of initiator caspases results in the downstream activation of effector caspases that cleave key cellular proteins leading to controlled cell death^[36]. To assess the involvement of caspases, we measured the proteolytic activity of the effector caspases-3/7 using a luminogenic substrate assay. The caspase-3/7 activity of untreated MC38 or Colon26 cells was normalized to 100%. As shown in Figure 5B, 36 h after transduction of MC38 or Colon26 cells with Ad.RSV-G or Ad.RSV-F_{sol}, the median caspase-3/7 activity was about 107%. Treatment with Ad.RSV-F or Ad.RSV-F/G resulted in about 142% caspase activity.

In addition, we analyzed the mitochondrial membrane potential $\Delta\Psi_m$, an important parameter of mitochondrial alterations, 36 h after transduction of the cells with the

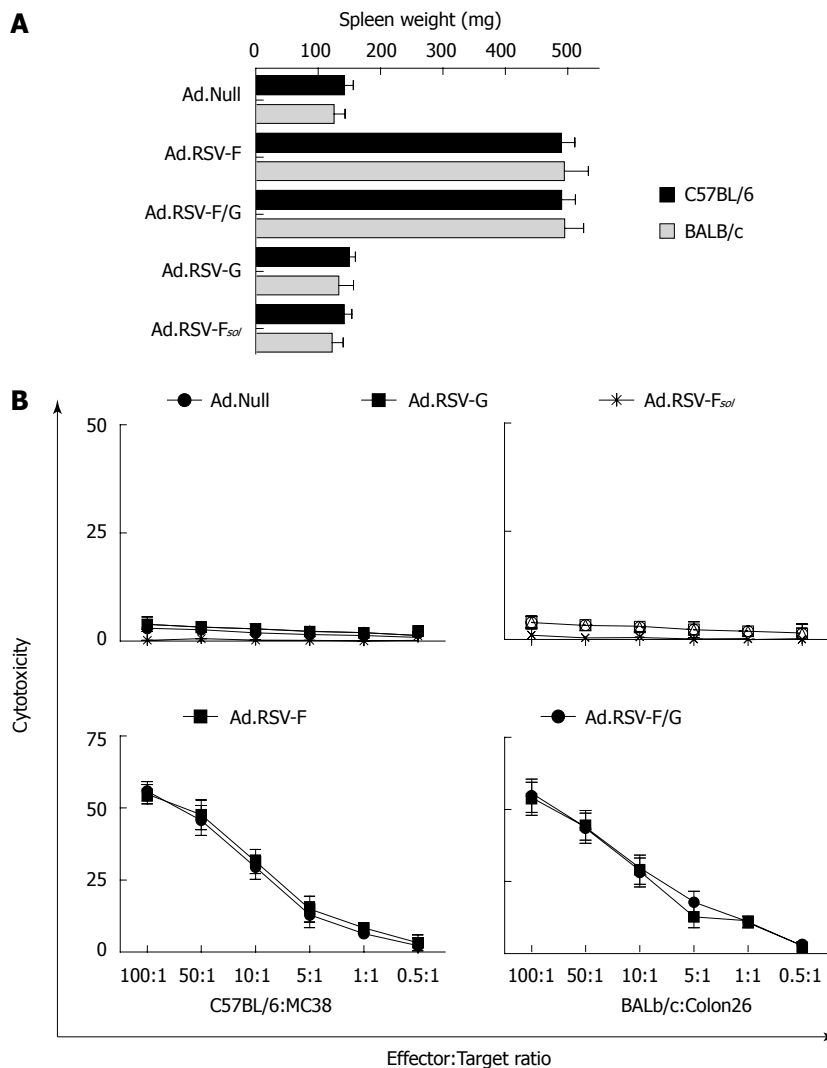


Figure 6 Effects of indicated treatments on the spleen weight and cytotoxic T cell induction. **A:** In the bilateral subcutaneous syngeneic MC38 and Colon26 tumor model described in Figure 2, animals were euthanized at day 28 and spleen weight was determined (mean \pm SD); **B:** In addition, we determined the cytotoxic activity of spleen-derived T cells against target MC38 or Colon26 tumor cells in these animals using an LDH release assay. Data of all animals were expressed as the average percentage of specific LDH release from three independent experiments (mean \pm SD).

adenoviral vectors, by staining with the cationic dye JC-1, which selectively enters into mitochondria and reversibly changes color from green to red as the membrane potential increases. In healthy cells with high mitochondrial $\Delta\Psi_m$, JC-1 spontaneously forms complexes with intense red fluorescence. In apoptotic or unhealthy cells with low $\Delta\Psi_m$, JC-1 remains in the monomeric form, which shows green fluorescence. As shown in Figure 5C, transduction of MC38 or Colon26 cells with Ad.RSV-F or Ad.RSV-F/G resulted in a clearly stronger loss of $\Delta\Psi_m$ compared with cells transduced with Ad.Null, Ad.RSV-G or Ad.RSV-F_{sol}.

Treatment of animals with Ad.RSV-F or Ad.RSV-F/G but not with Ad.RSV-G or Ad.RSV-F_{sol} results in significantly increased spleen weight

Splenomegaly is often associated with a cellular immune response. To elucidate whether the anti-neoplastic effect on the untreated contralateral tumor is immune-mediated we determined the spleen weights of the animals on d 28 (Figure 6A). We observed about a 245% increase in median spleen weights in the MC38 and Colon26 cancer model animals treated with Ad.RSV-F or Ad.RSV-F/G, compared to Ad.Null-treated animals. Treatment of animals with Ad.Null, Ad.RSV-G or Ad.RSV-F_{sol} had no significant effect on spleen weight.

Intratumoral expression of RSV-F or RSV-F/G but not of RSV-G or RSV-F_{sol} induces a tumor cell-specific CTL response

As the splenomegaly suggests the induction of a cellular immune response, we next determined whether there is a tumor-specific CTL response mediating the observed anti-neoplastic response against the untreated tumor. As shown in Figure 6B, in an LDH release assay we observed no cytotoxicity of splenocytes derived from Ad.Null-treated mice with or without tumors against the MC38 or Colon26 target cells. Splenocytes from animals treated with Ad.RSV-F or Ad.RSV-F/G showed a cytotoxicity of about 55% at an effector to target ratio of 100:1. By contrast, we observed only a slight lysis of target cells by splenocytes from animals treated with Ad.RSV-G or Ad.RSV-F_{sol}.

DISCUSSION

We demonstrated previously in syngeneic murine tumor models that intratumoral expression of viral fusogenic membrane proteins can induce a systemic anti-tumor response associated with a tumor-specific CTL response^[26,37]. In this study we analyzed whether *in situ* tumor vaccination by intratumoral expression of RSV envelope glycoproteins is influenced by the efficiency

of cell-cell fusion, the mode of tumor cell death, or whether the effect is mediated by intrinsic immunological properties of the RSV-F protein.

The key findings of our current study were as follows. First, despite the limited intratumoral spread and transduction efficiency of the replication-defective adenovirus vectors^[38], intratumoral injection of Ad.RSV-F or Ad.RSV-F/G resulted in significantly improved tumor growth reduction of both vector-inoculated tumors and contralateral untreated tumors. The improved anti-neoplastic treatment efficacy also resulted in a significantly improved survival. The effect was not mediated by an adenovirus vector viremia, as we did not observe this effect in nude mice (data not shown) and we did not detect adenovirus vector beyond d 1 in the serum of vector-treated C57BL/6 mice^[39]. This indicates that the systemic anti-tumor response depends on an intact immune system. This is supported by our demonstration of the induction of a tumor-specific CTL response and a massively increased spleen weight. This qualitatively confirms our data obtained in a bilateral subcutaneous colon cancer model using HSV-1 or adenovirus vectors encoding the fusogenic membrane proteins F and H of measles virus or RSV-F, showing that the expression of viral fusogenic membrane proteins can serve as a tumor vaccination platform^[25,26,37]. Furthermore the data are in concert with previous studies demonstrating that expression of VSV-G encoded by a plasmid vector can enhance the immunogenicity of tumor cells^[17,40,41].

Second, we demonstrated that transduction of tumor cells with Ad.RSV-F/G resulted in clearly larger syncytia than transduction with Ad.RSV-F. This result is in concert with a previous observation^[42]. However, as we used codon-optimized cDNA for the expression of the RSV glycoproteins^[29], we observed cell-cell fusion also after transduction of the tumor cells with Ad.RSV-F alone. In other paramyxoviruses, expression of the fusion protein alone is not sufficient to mediate cell-cell fusion^[43]. Transduction of the murine colon cell lines with Ad.RSV-G or Ad.RSV-F_{sol} did not cause detectable cell-cell fusion.

Third, there was no significant difference in the local and distant anti-tumor effect in animals that were treated with Ad.RSV-F or Ad.RSV-F/G, although the syncytia of cells transduced with Ad.RSV-F/G were clearly larger *in vitro*. This indicates that the treatment outcome was not significantly influenced by the size of cell-cell fusion.

Fourth, our data indicate that only RSV envelope glycoproteins which cause syncytium formation (RSV-F or RSV-F/G) are able to induce apoptosis, in contrast to RSV-G or RSV-F_{sol}. Importantly, in our experimental setting, only membrane proteins which are able to induce apoptosis are associated with a local and distant anti-tumor effect. Because the expression of RSV-F_{sol} did not induce a distant anti-tumor response, this effect is most likely not mediated by intrinsic immunological properties of RSV-F. This supports previous data indicating the mechanisms by which tumor cells are killed may be critical for the induction of a specific anti-tumor immunity^[44,45]. However, because we did not include a fusion-disabled RSV-F, we cannot rule out the possibility that the transmembrane

domain of RSV-F *per se* is responsible for the observed effects.

In an earlier study we elucidated by Western blot analysis some of the molecular pathways leading to cell death by expression of viral fusogenic membrane proteins. We demonstrated that induction of apoptosis was independent of functional p53 and was mediated *via* a mitochondrial death pathway triggered by modulation of Bcl-2 family proteins^[32]. In addition, we demonstrated increased protein levels of the heat shock proteins (HSP) 60, 70 and 90 α ^[46].

According to the danger model, growing tumors do not provide a danger signal to dendritic cells, and thus, do not activate the immune system. Therefore, any tumor antigen-specific T cell will have its first antigen encounter with tumor cells or resting dendritic cells. Because there is no co-stimulation, either situation will drive the T cells into anergy or apoptosis and, eventually, tumor tolerance^[47]. A conceivable mechanism for the induction of tumor-specific immunity is that expression of the viral fusogenic membrane proteins, apoptotic cells or exosomes of fused cells^[48,49] might release danger signals resulting in a more efficient presentation of tumor antigens and activation of T cells. This is in concert with recent reports demonstrating that apoptotic tumor cells, but not malignant cells in necrotic tumors, can provoke an anti-tumor immune response^[50], if the tumor cells were killed in a caspase-3-dependent manner^[51]. In addition, the xenogenization of tumor cells by presentation of viral antigens on the cell surface in conjunction with MHC class I molecules might contribute to the induction of a tumor-specific immune response^[52,53].

In this study we demonstrated that enhanced fusion function of RSV-F by co-expression of RSV-G does not significantly enhance *in situ* tumor vaccination in the two tested syngeneic tumor models. However, our data indicate that the vaccination effect critically depends on tumor cell apoptosis, which can be further enhanced by combination with chemotherapy and viral oncolysis^[32,37]. Overall, these findings provide insight into improved tumor vaccination strategies relying on the intratumoral expression of viral fusogenic membrane proteins.

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COMMENTS

Background

In 1994, Polly Matzinger presented a new theory called the danger model, suggesting that a specific immune response develops as a result of danger detection rather than discrimination between self and non-self antigens. According to the danger model, the immune surveillance system fails to detect tumor antigens because transformed cells do not send any danger signals. Danger

signals are thought to act by stimulating dendritic cells to mature so that they can present foreign antigens and stimulate T cells. Dying mammalian cells have also been found to release danger signals. In the absence of such signals there is often no immune response or tolerance may develop.

Research frontiers

Linardakis *et al* demonstrated in a syngeneic murine B16 melanoma model that the expression of fusogenic membrane protein G from vesicular stomatitis virus (VSV-G) can enhance the efficacy of a weak allogeneic vaccine. Hoffmann *et al* demonstrated previously that expression of viral fusogenic membrane proteins can induce apoptosis in tumor cells.

Innovations and breakthroughs

In two syngeneic subcutaneous murine colon cancer models we demonstrated that intratumoral injection of a replication-defective adenovirus encoding respiratory syncytial virus fusion protein (RSV-F) alone (Ad.RSV-F) or together with the attachment glycoprotein RSV-G (Ad.RSV-F/G) leads to a significant growth reduction of both the vector-treated and contralateral untreated tumor. Treatment response was associated with a strong tumor-specific CTL response and significantly improved survival. Intratumoral injection of Ad.RSV-G or a soluble RSV-F encoding adenovirus (Ad.RSV-F_{sol}) had no significant anti-neoplastic effect, suggesting the therapeutic effect is not mediated by intrinsic immunological properties of the viral proteins. Although *in vitro* transduction of colon cancer cell lines with Ad.RSV-F/G resulted in about 8-fold larger syncytia than with Ad.RSV-F, the *in vivo* outcome was not significantly different. Transduction of murine colon cancer cell lines with Ad.RSV-F or Ad.RSV-F/G caused apoptotic cell death, in contrast to Ad.RSV-G or Ad.RSV-F_{sol}, suggesting an importance of the mode of cell death. Our results provide insight into improved tumor vaccination strategies relying on the intratumoral expression of viral fusogenic membrane proteins.

Applications

This strategy might be applicable for the treatment of human cancer.

Peer review

This is a good study designed to elucidate the relevance of the size of syncytia or the way tumor cells die on the therapeutic outcome after tumor vaccination using intratumoral expression of viral fusogenic membrane proteins. The results are informative and potentially helpful for human cancer treatments.

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Effect of JIANPI HUOXUE decoction on inflammatory cytokine secretion pathway in rat liver with lipopolysaccharide challenge

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Abstract

AIM: To evaluate the effect of Chinese traditional medicinal prescription, JIANPI HUOXUE decoction (JHD) on cytokine secretion pathway in rat liver induced by lipopolysaccharide (LPS).

METHODS: Twenty-four male SD rats were divided into normal group ($n = 4$), model group ($n = 10$) and JHD group ($n = 10$) randomly. Rats in model group and JHD group were administrated with normal saline or JHD *via* gastrogavage respectively twice a day for 3 d. One hour after the last administration, rats were injected with LPS *via* tail vein, 50 $\mu\text{g}/\text{kg}$. Simultaneously, rats in normal group were injected with equivalent normal saline. After LPS stimulation for 1.5 h, serum and liver tissue were collected. Pathological change of liver tissues was observed through hematoxylin-eosin (H.E.) staining. Tumor necrosis factor alpha (TNF- α) in serum were assayed by enzyme linked immunosorbent assay (ELISA). The protein expression of TNF- α , phosphorylated inhibit- κB (p-I κB) and CD68 in liver were assayed by Western blot. The distribution of CD68 protein in liver was observed through immunohistochemical staining. The mRNA expression of TNF- α , interleukin-6 (IL-6), CD14, toll-like receptor 2 (TLR2) and TLR4 in liver were assayed by real-time RT-PCR.

RESULTS: Predominant microvesicular change, hepatocyte tumefaction and cytoplasm dilution were observed in liver tissues after LPS administration as well as obvious CD68 positive staining in hepatic sinusoidal. After LPS stimulation, serum TNF- α (31.35 ± 6.06 vs 12225.40 ± 9007.03 , $P < 0.05$), protein expression of CD68 (1.13 ± 0.49 vs 3.36 ± 1.69 , $P < 0.05$), p-I κB (0.01 ± 0.01 vs 2.07 ± 0.83 , $P < 0.01$) and TNF- α (0.27 ± 0.13 vs 1.29 ± 0.37 , $P < 0.01$) in liver and mRNA expression of TNF- α (1.96 ± 2.23 vs 21.45 ± 6.00 , $P < 0.01$), IL-6 (4.80 ± 6.42 vs 193.50 ± 36.36 , $P < 0.01$) and TLR2 (1.44 ± 0.62 vs 4.16 ± 0.08 , $P < 0.01$) in liver were also increased significantly. These pathological changes were all improved in JHD group. On the other hand, TLR4 mRNA (1.22 ± 0.30 vs 0.50 ± 0.15 , $P < 0.05$) was down-regulated and CD14 mRNA increased but not significantly after LPS stimulation.

CONCLUSION: JHD can inhibit cytokine secretion pathway induced by LPS in rat liver, which is probably associated with its regulation on CD68, p-I κB and endotoxin receptor TLR2.

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Key words: JIANPI HUOXUE decoction; Lipopolysaccharide; Kupffer cell; Cytokine; Endotoxin receptor

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INTRODUCTION

When activated under physiologically challenging

conditions, such as endotoxemia or immune reactions, macrophages release large amounts of cytokines, interleukins and prostanoids, which may result in organ damage. Tumor necrosis factor alpha (TNF- α) is a potent inflammatory cytokine which can exert a variety of effects on cells ranging from mitochondrial damage and oncotic or apoptotic necrosis to cell proliferation^[1]. TNF- α may also prompt the accumulation of neutrophils (PMNs) by activating endothelial cells^[1,2]. It can indirectly promote toxicity by priming PMNs to release reactive oxygen and nitrogen species and proteases that damage nearby cells^[2,3]. An overproduction of TNF- α is associated with the development of alcoholic liver injury^[4-6]. Kupffer cells, the resident macrophages in the liver, are the major producers of TNF- α following exposure to lipopolysaccharide (LPS), the bacterial endotoxin^[7], and play an important role in alcohol-induced liver damage^[8]. It has been demonstrated that Kupffer cell-derived cytokines induced by intestinal-derived LPS involves in the mechanism of alcoholic liver disease (ALD)^[8-11].

Multiple mammalian receptors for LPS have been identified, including two glycoproteins: LPS-binding protein (LBP) and CD14. CD14 binds to the LPS-LBP complex and interact with a transmembrane toll-like receptor (TLR) responsible for signal transduction^[12]. TLR4 is a specific receptor for gram-negative bacterial LPS^[13]. Several observations indicate that TLR2 is also involved in LPS signaling^[14-19].

JIANPI HUOXUE decoction (JHD) consists of eight Chinese herbs. Previously, JHD was found to inhibit endotoxin levels and intestine or liver injury in ALD rats induced by Lieber-DeCarli ethanol liquid diet^[20,21]. In the present study, we have isolated the LPS-induced cytokine secretion pathway from the complex conditions of ALD by employing the LPS challenging model described previously^[22] to further explore the effects of JHD on this pathway. This might provide a new point of view on the mechanisms of JHD anti-alcoholic liver injury.

MATERIALS AND METHODS

JHD preparation

JHD consists of *Altractylodes macrocephala* Koidz., *Salvia miltiorrhiza* Bge., *Citrus aurantium* L., *Paeonia lactiflora* pall., *Pueraria lobata* (willd.) Ohwi, *Alisma orientalis* (Sam.) Juzep., *Schisandra chinensis* (Turcz.) Baill. and *Curuma longa* L. *Altractylodes macrocephala* Koidz., *Citrus aurantium* L. and *Curuma longa* L. were distilled with ethanol to get the volatilizable components for three times, each lasting 1-2 h. *Schisandra chinensis* (Turcz.) Baill. was also extracted with ethanol twice, independently, 1-2 h for each time. The other herbs were boiled with water for three times after being marinated in water for 1 h. The final density of the water-extraction was 1.08-1.12 (80°C) and the water-extraction was purified with ethanol. Finally, the volatilized components, ethanol-extraction of *Schisandra chinensis* (Turcz.) Baill. and water-extraction were mixed as the JHD. The concentration of 0.9 g crude drug/mL was used in experiments.

Animal and treatment

Twenty-four female SD rats weighing 200 g were divided randomly into normal group ($n = 4$), model group ($n = 10$) and JHD group ($n = 10$). Rats in model group and JHD group were administrated with normal saline or JHD *via* gastrogavage respectively, 5 mL/kg, twice a day, for 3 d. One hour after the last administration, rats in these two groups were injected with LPS (*Escherichia coli* 0111: B4, Sigma-Aldrich Co., USA) *via* tail vein, 50 μ g/kg body weight as described previously^[21]. Simultaneously, rats in normal group were injected with equivalent volume of normal saline. After LPS stimulation for 1.5 h, serum and liver tissue were collected for assay.

Histological examination

Sections of the liver sample (4 μ m thick) were stained with hematoxylin-eosin (H.E.) and examined under light microscope (Olympus Medical Systems Corp., Tokyo, Japan).

Immunohistochemical assessment of CD68

Specimens were fixed in a 40 g/L solution of formaldehyde in 0.1 mol/L phosphate-buffered saline (pH 7.4) and embedded in paraffin wax from which 4 μ m thick sections were taken on a slide coated with poly-L-lysine (Dingguo Ltd., Beijing, China) for immunohistochemical assessment. Antigen retrieval was performed with 0.6% pepsin (Dingguo Ltd., Beijing, China) at 37°C for 10 min. The specimens were treated with 0.6% hydrogen peroxide-methanol, following 30 min of endogenous peroxidase blockage at 37°C. After blockage with 0.2% bovine serum albumin (BSA, Sino-American Biotechnology Company, Shanghai, China) for 20 min at room temperature, the samples were incubated at 4°C overnight, with a 1:100 dilution of anti-CD68 primary antibody (monoclonal anti-rat CD68, AbD Serotec, NC, USA). Following the processing of the samples incubated with a 1:250 dilution of horseradish peroxidase (HRP)-linked goat anti-mouse IgG (sc-2031, Santa Cruz Biotechnology Inc. Santa Cruz, CA) for 1 h at 37°C, diaminobenzidine (DAB) was applied as a chromogen and hematoxylin was used for floor staining.

Measurement of serum TNF- α by ELISA

Serum levels of TNF- α were determined using a commercially available enzyme linked immunosorbent assay (ELISA) kit (Biosource International Inc., Camarillo, CA) according to the manufacturer's instruction. TNF- α was determined from a standard curve for the combination of these cytokines. The concentrations were expressed as pg/mL.

Determination of CD68, phosphorylated inhibit- κ B (p-I κ B) and TNF- α level in liver tissue by Western blot

As described previously^[23], total protein was extracted from liver tissue and analyzed with bicinchoninic acid (BCA) protein concentration assay kit (Beyotime Inst. Biotechnology, Jiangsu, China). Sample protein was separated by electrophoresis in 10% SDS-PAGE separating gel with Bio-Rad electrophoresis system (Bio-

Rad Laboratories, Hercules, CA, USA). The primary antibodies (mouse anti-rat glyceraldehydes-3-phosphate dehydrogenase, GAPDH antibody, 1:5000 dilution, KANGCHEN Bio-Tech Inc., Shanghai, China; mouse anti-rat CD68 antibody, 1:100 dilution, AbD Serotec, NC, USA; mouse anti-rat p-IkB antibody, 1:500 dilution, Cell Signaling Technology Inc., Boston, USA; goat anti-rat TNF- α antibody, 1:500 dilution, R&D Systems Inc., Minneapolis, USA) were incubated at 4°C overnight. The corresponding horseradish peroxidase-conjugated secondary antibodies (goat anti-mouse IgG, peroxidase-linked antibody, 1:5000 dilution, Santa Cruz Biotechnology Inc., Santa Cruz, CA, rabbit anti goat-IgG, peroxidase-linked antibody, 1:5000 dilution, Jackson ImmunoResearch Laboratories Inc., PA, USA) were incubated at room temperature. The ECL kit (Pierce Biotechnology Inc., Rockford, USA) and the Furi FR-980 image analysis system (Shanghai Furi Co., Shanghai, China) were employed for revealing and quantitative analysis of the blots. GAPDH protein was used as the internal control.

Determination of TNF- α , interleukin-6 (IL-6), TLR2, TLR4 and CD14 mRNA levels by real-time RT-PCR

Total RNA was extracted from liver tissues of each group with the tissue/cell total RNA isolation kit (Watson Biotechnologies Inc., Shanghai, China) according to the manufacturer's protocol. The quantity and purity of RNA were detected by determining absorbance at 260/280 nm using a spectrophotometer (Unico Co., USA). Total RNA was reversely transcribed into complementary DNA (cDNA) using the cDNA synthesis kit (Fermentas Life Sciences Inc., Maryland, USA) according to the manufacturer's protocol. The Rotor Gene-3000 PCR machine (Gene Co., Hong Kong) and real-time PCR kit (SYBR® Premix Ex Taq™, TaKaRa Bio Inc., Japan) were employed based on the manufacturer's instruction. The specific primers for the target genes and β -actin (synthesized by Shanghai Sheng Gong Co.) used are described in Table 1.

Two-step PCR procedure was recommended as follows: pre-denaturation for 10 s at 95°C, 1 cycle; 95°C for 5 s and 59°C for 20 s, 40 cycles. The final products were identified by electrophoresis in 1.5% agarose gel and melt curve analysis. Two-standard curve method was employed in relative quantification analysis. Briefly, after the target gene products were emendated with internal control β -actin, the relative fluorescence values of target products in normal group were analysed and compared with other groups. The final results were described with the relative values. The calculation and analysis were performed by the software in the Rotor-Gene RG-3000 (Gene Co., Hong Kong).

Statistical analysis

All values were expressed as mean \pm 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 JHD on pathological changes of liver tissue in rats

Table 1 Primers for the target genes and β -actin used in real-time PCR

Target gene	Primer	Target fragment length (bp)
β -actin	5'-TGACGAGGCCAGAGCAAGA-3'(F) 5'-ATGGGCACAGTGTGGGTGAC-3'(R)	331
TNF- α	5'-GGCAGCCTTGTCCTTGAAGAG-3'(F) 5'-GTAGCCACGTCGTAGCAAACC-3'(R)	171
IL-6	5'-CCACTTCACAAGTCGGAGGCTTA-3'(F) 5'-GTGCATCATCGCTGTCATACAATC-3'(R)	108
TLR4	5'-CTCACAACITCAGTGGCTGGATTTA-3'(F) 5'-TGTCTCCACAGCCACCAGATTC-3'(R)	178
TLR2	5'-GGCCACAGGACTCAAGAGCA-3'(F) 5'-AGAGGCCTATCACAGCCATCAAG-3'(R)	102
CD14	5'-GAATCCCAGTCGGAGGCGTA-3'(F) 5'-GGAGCAAAGCCAAAGTTCCTGA-3'(R)	94

H.E. staining showed predominant microvesicular change, hepatocyte tumefaction and cytoplasm dilution after LPS stimulation in the rat liver tissues. After JHD administration, those pathological changes of liver tissues were ameliorated obviously (Figure 1 A1-A3).

Effect of JHD on CD68 protein expression and distribution in liver tissue

CD68 immunohistochemical staining indicated that a spot of positive staining existed in hepatic sinusoidal of normal rats and obvious positive staining in the sinusoidal where hepatic microvesicular change was predominant after LPS stimulation. After JHD administration, the CD68 positive staining in the sinusoidal was lightened (Figure 1 B1-B3).

Effect of JHD on serum TNF- α level of rats

After stimulation with LPS for 1.5 h, serum TNF- α increased significantly (31.35 ± 6.06 vs 12225.40 ± 9007.03 , $P < 0.05$) and decreased obviously in JHD group (12225.40 ± 9007.03 vs 6031.70 ± 2296.56 , $P < 0.05$) (Figure 2).

Effect of JHD on protein expression of CD68, p-IkB and TNF- α in liver tissues

After stimulation with LPS for 1.5 h, protein expression of CD68, p-IkB and TNF- α in liver tissues were increased significantly (CD68: 1.13 ± 0.49 vs 3.36 ± 1.69 , $P < 0.05$; p-IkB: 0.01 ± 0.01 vs 2.07 ± 0.83 , $P < 0.01$; TNF- α : 0.27 ± 0.13 vs 1.29 ± 0.37 , $P < 0.01$) and decreased significantly (CD68: 3.36 ± 1.69 vs 0.76 ± 0.45 , $P < 0.05$; p-IkB: 2.07 ± 0.83 vs 0.87 ± 0.83 , $P < 0.01$; TNF- α : 1.29 ± 0.37 vs 0.67 ± 0.36 , $P < 0.01$) in JHD group (Figure 3).

Effect of JHD on mRNA expression of TNF- α , IL-6, TLR2, TLR4 and CD14 in liver tissues

After stimulation with LPS for 1.5 h, mRNA expression of TNF- α , IL-6 and TLR2 in liver were increased significantly (TNF- α : 1.96 ± 2.23 vs 21.45 ± 6.00 , $P < 0.01$; IL-6: 4.80 ± 6.42 vs 193.50 ± 36.36 , $P < 0.01$; TLR2: 1.44 ± 0.62 vs 4.16 ± 0.08 , $P < 0.01$) and decreased obviously in JHD group (TNF- α : 21.45 ± 6.00 vs 11.99 ± 2.28 , $P < 0.01$; IL-6: 193.50 ± 36.36 vs 76.12 ± 32.16 , $P < 0.01$; TLR2: 4.16 ± 0.08 vs 3.11 ± 0.53 , $P < 0.01$).

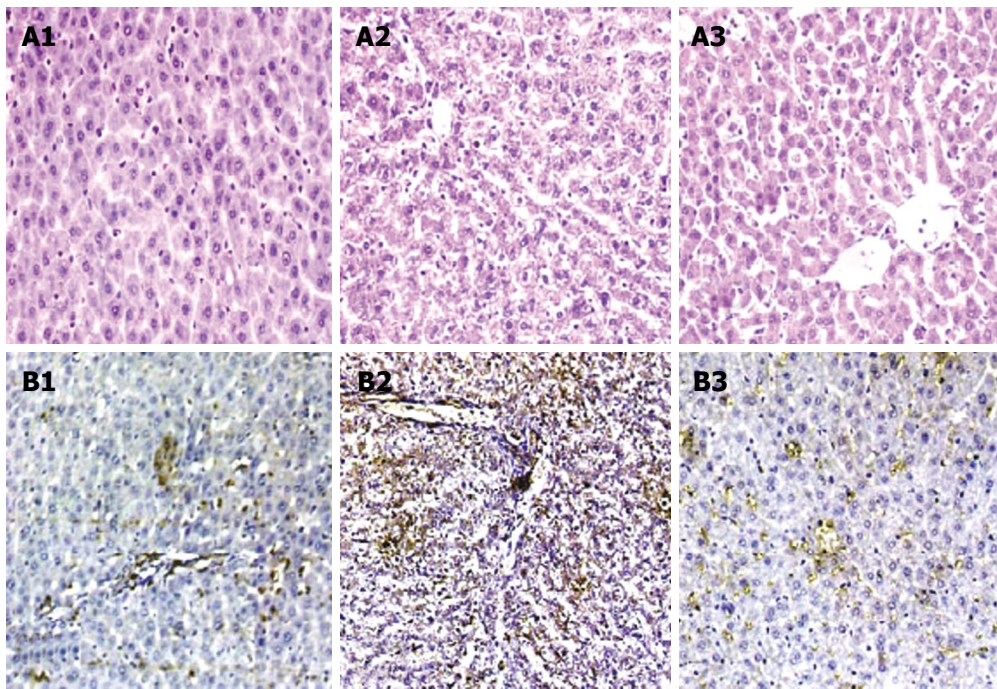


Figure 1 Effects of JHD on pathological changes and CD68 protein expression and distribution in liver tissue. **A:** HE stain ($\times 400$); **A1:** Liver tissue in normal group; **A2:** Liver tissue in model group; **A3:** Liver tissue in JHD group; **B:** CD68 immunohistochemical stain ($\times 200$); **B1:** Liver tissue in normal group; **B2:** Liver tissue in model group; **B3:** Liver tissue in JHD group.

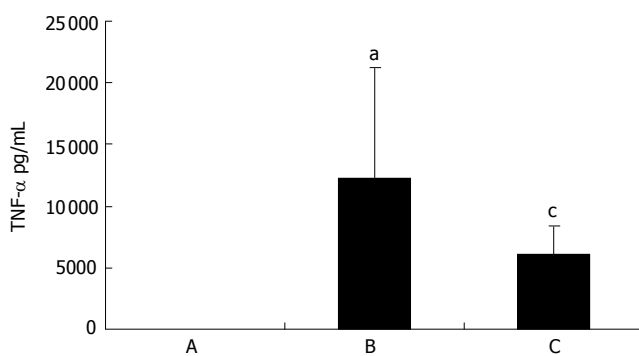


Figure 2 Effect of JHD on serum TNF- α level of rats. **A:** Normal group; **B:** Model group; **C:** JHD group. $^aP < 0.05$ vs A; $^cP < 0.05$ vs B.

On the other hand, TLR4 mRNA expression was down-regulated (1.22 ± 0.30 vs 0.50 ± 0.15 , $P < 0.05$) and mRNA expression of CD14 was increased but not significantly after LPS stimulation for 1.5 h (Figure 4).

DISCUSSION

Gut-derived LPS is the primary endogenous endotoxin of gram-negative bacteria^[9]. Extended ethanol exposure can lead to gut leakage^[20] and the gut-derived LPS consequently enter the circulation. Previous researches have confirmed that LPS promoted the phosphorylation of inhibit- κ B ($\text{I}\kappa\text{B}$), transferred the NF- κ B into nuclear, consequently promoted cytokines production in Kupffer cells, which was involved in the pathogenesis of ALD^[9-12,24,25].

In the present research, the predominant microvesicular change, hepatocyte tumefaction and cytoplasm dilution were observed in liver tissues after LPS stimulation, and simultaneously, CD68, the specific antigen-molecule on the microphage, was identified predominantly in the sinusoidal. The protein and/or gene expression of

inflammatory factors, such as TNF- α and IL-6, and the protein expression of CD68 and p-I κ B were also increased by LPS stimulation. On the contrary, JHD inhibited the pathological changes significantly.

The endotoxin receptors are necessary for LPS signal transferring. CD14, TLR4 and TLR2 have been identified as the main endotoxin receptors^[12-19].

Soluble CD14 (sCD14) exists in blood and membrane CD14 (mCD14) anchors on the membrane of peripheral or liver resident microphage (Kupffer cell) through glycosylphosphatidylinositol (GPI). LPS-LBP complex combines with mCD14 and activated cells through TLR4^[27]. CD14 has also been found to bind lipoteichoic acid (LTA) and peptidoglycan (PGN) from the cell wall of gram-positive bacteria and activate cells through TLR2^[27]. The expression of CD14 stimulated by LPS was reported as a dynamic process. C Fearn *et al*^[28] found that 1 h after intraperitoneal injection of LPS, CD14 mRNA was induced in the liver of mice but not significantly, and peaked at 8-16 h. By 24 h, the level of CD14 mRNA returned to the basal level. In the present study, we also found that 1.5 h after injection of LPS *via* tail vein, the level of CD14 mRNA increased but not significantly, which might suggest that at this time point, CD14 gene level did not reach the peak in this model.

TLR4 has been proved to be the specific receptor for the LPS of gram-negative bacteria^[11,29]. The reports on TLR4 mRNA expression stimulated by LPS were not consistent. Matsumura T *et al*^[18] found when mice were administered LPS, TLR4 mRNA was decreased in the brain, increased in the heart and lung and not affected in the liver, kidney, and spleen. In the study by Choda Y *et al*^[30], TLR4 mRNA level was increased 0.5 h after intraportal LPS administration, but decreased thereafter. Liu XW *et al*^[31] found that 3 h after intraperitoneal injection LPS in

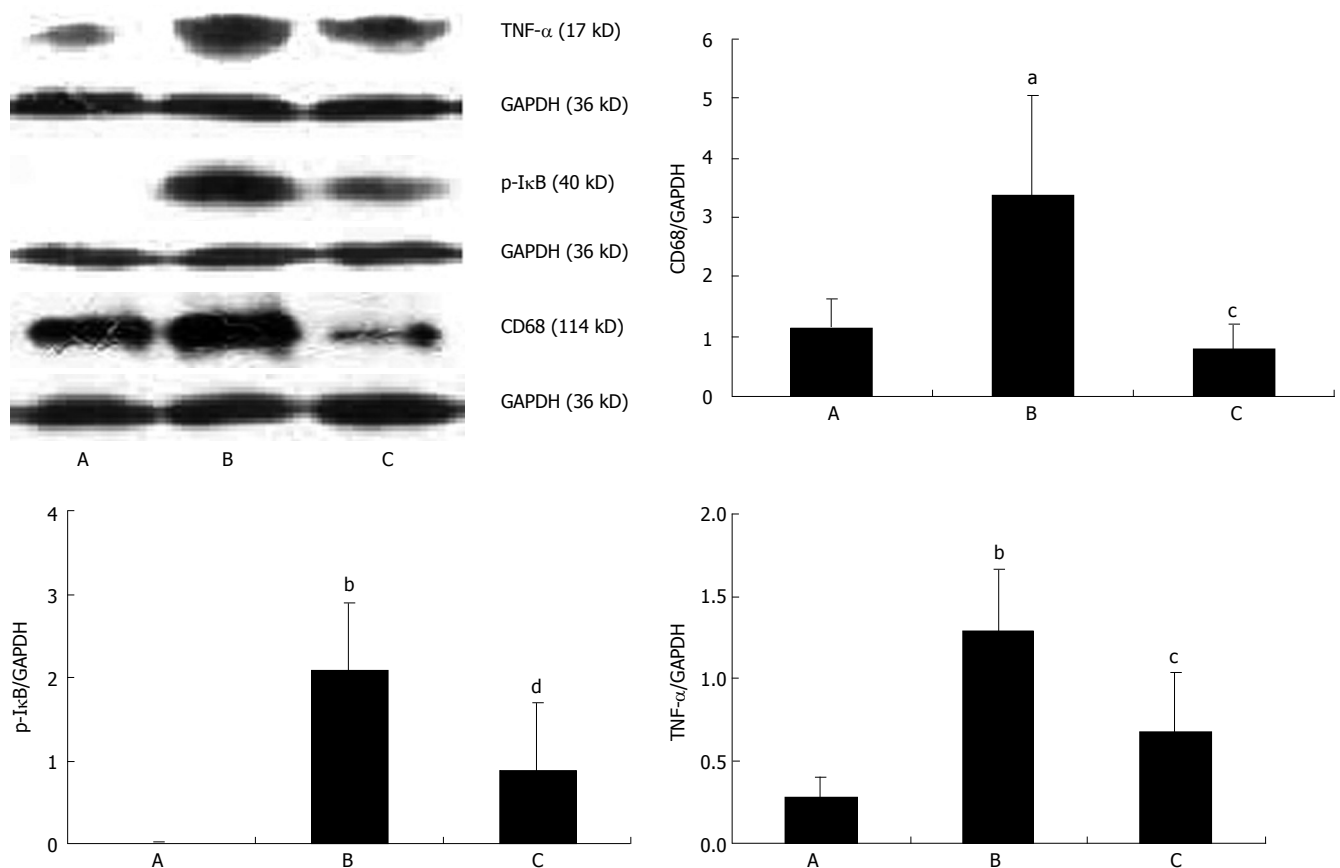


Figure 3 Effect of JHD on protein expression of CD68, p-IκB, TNF-α in liver tissue. A: Normal group; B: Model group; C: JHD group. ^a $P < 0.05$ vs A; ^b $P < 0.01$ vs A; ^c $P < 0.05$ vs B; ^d $P < 0.01$ vs B.

mice, *TLR4* mRNA was down-regulated in liver and failed to be detected by 6–12 h, but resumed to the basal level by 24 h. In our research, significant decrease of *TLR4* mRNA expression was observed in liver 1.5 h after LPS administration, which was consistent with the report by Y Choda.

The primary ligands of TLR2 are gram-positive bacteria-derived lipoteichoic acid (LTA), peptidoglycan (PGN) and mycobacterial lipoarabinomannan^[32]. Previous reports suggested that neither human nor murine TLR2 plays a role in LPS signaling^[15,33]. But the subsequent research^[34] found that phenol repurified LPS did not activate cells from TLR2-mediated signaling, but the commercially prepared LPS contained low concentrations of highly bioactive contaminants described previously as endotoxin protein activated TLR2-mediated signaling. On the other hand, myeloid differentiated protein-2 (MD-2) which is necessary for TLR4-mediated LPS signaling was also proved to enable TLR2 to respond to endotoxin protein-free LPS and enhance TLR2-mediated responses to both gram-negative bacteria and their LPS^[16]. Furthermore, the cytokines induced by LPS, such as interleukin-1 beta (IL-1β) or TNF-α, also up-regulate *TLR2* mRNA of rat hepatocyte *in vivo* and *in vitro*^[17]. As a result, LPS from gram-negative bacteria does not induce TLR2 expression directly, but induced the commercial LPS containing endotoxin protein indirectly. LPS-derived

cytokine and MD-2 can induce TLR2 expression greatly. Our results also suggested that *TLR2* mRNA was up-regulated significantly in liver after LPS administration. And as expected, *TLR2* mRNA in JHD group decreased significantly.

In conclusion, the present study confirmed that the inhibitory effects of JHD on cytokines (TNF-α, IL-6) protein or gene expression induced by LPS in liver is associated with its inhibition on the LPS-activated Kupffer cell signal pathway, including CD68 and p-IκB protein expression and *TLR2* mRNA expression.

COMMENTS

Background

The "two-hits" theory brought about great advancement in pathogenesis research of alcoholic liver disease (ALD). Attention has been attracted in the mechanism of endotoxin or lipopolysaccharides (LPS) signal pathway involved in ALD. The resident macrophage in liver, Kupffer cells, is an important target cell of LPS and secreting cytokines. The endotoxin receptors, identified as CD14, toll-like receptor (TLR) 4, are essential for LPS signaling.

Research frontiers

In this study, the expression of endotoxin receptors, CD14, TLR4 and TLR2 was observed after LPS or LPS + JHD administration. The difference of endotoxin receptors expression under similar challenge conditions is interesting, especially, TLR4 and CD14.

Innovations and breakthroughs

This study confirmed one of the possible mechanisms of JHD, a Chinese

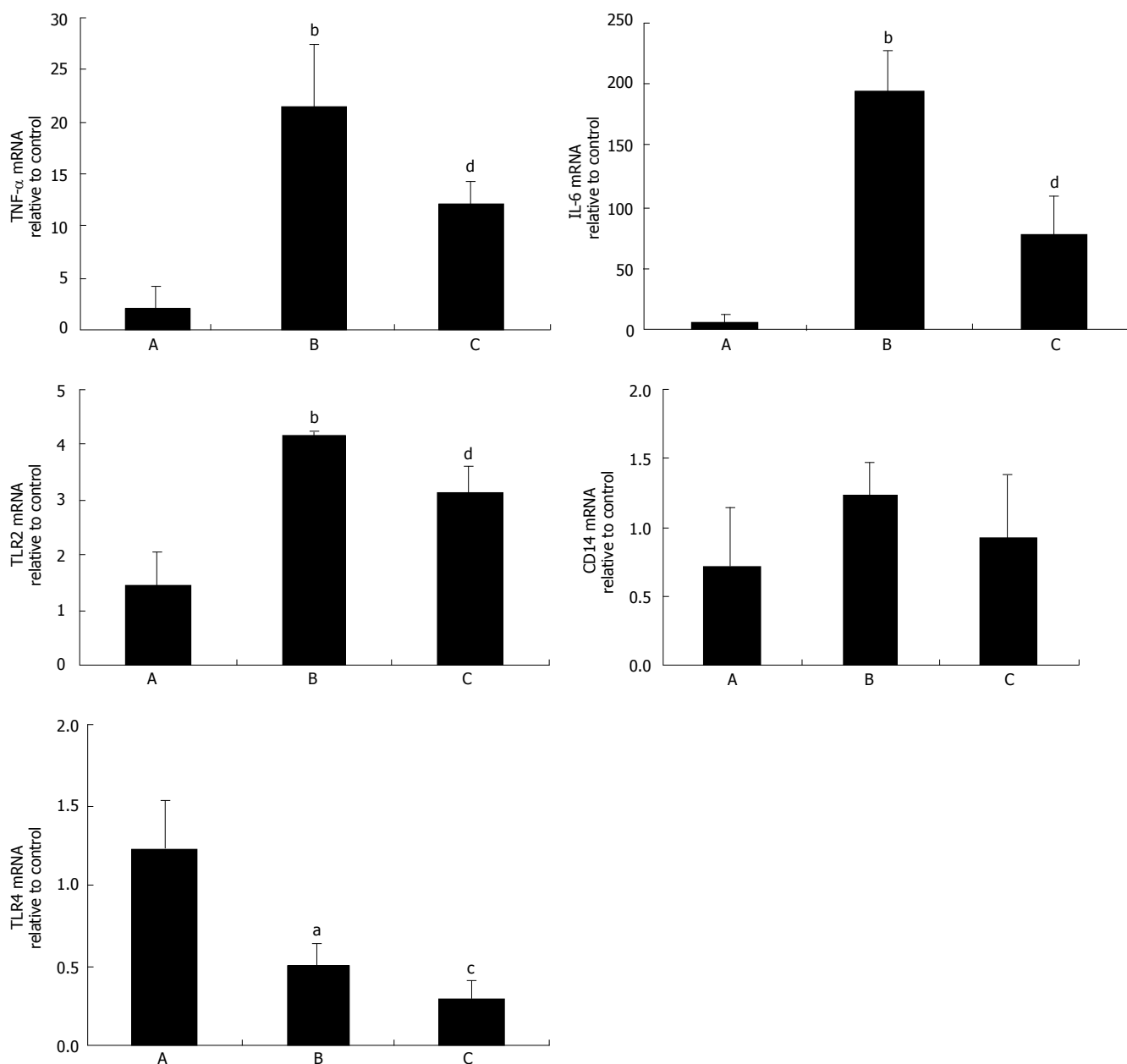


Figure 4 Effect of JHD on mRNA expression of TNF- α , IL-6, TLR2, TLR4, CD14 in liver tissue. A: Normal group; B: Model group; C: JHD group. ^a $P < 0.05$ vs A; ^b $P < 0.01$ vs A; ^c $P < 0.05$ vs B; ^d $P < 0.01$ vs B.

herbs decoction, on anti-alcoholic liver injury, inhibiting some targets in LPS-activated cytokine secretion pathway, such as CD68, the specific molecular marker of macrophage, phosphorylated inhibit- κ B (p-I κ B) and TLR2, the endotoxin receptor.

Applications

The present study provides a new experimental evidence of JHD inhibition alcoholic liver injury. To further evaluate the effects of JHD on Kupffer cell activation and endotoxin receptors expression induced by LPS, the multiple-time point observation and isolated Kupffer cells should be employed in the subsequent experiments.

Terminology

Lipopolysaccharide (LPS), the major composition of gram-negative bacterial wall, can excite intensive inflammation reaction.

Peer review

This is an interesting paper, describing the effects of a traditional Chinese medicine on the LPS activated Kupffer cells of murine liver. Methodology used is sound, results are well presented and analyzed, and conclusions are documented

by the findings of the study.

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BASIC RESEARCH

Over-expressed and truncated midkines promote proliferation of BGC823 cells *in vitro* and tumor growth *in vivo*

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Abstract

AIM: To determine whether midkine (*MK*) and its truncated form (*tMK*) contribute to gastric tumorigenesis using *in vitro* and *in vivo* models.

METHODS: Human *MK* and *tMK* plasmids were constructed and expressed in BGC823 (a gastric adenocarcinoma cell line) to investigate the effect of over-expressed *MK* or *tMK* on cell growth and tumorigenesis in nude mice.

RESULTS: The growth of *MK*-transfected or *tMK*-transfected cells was significantly increased compared with that of the control cells, and *tMK*-transfected cells grew more rapidly than *MK*-transfected cells. The number of colony formation of the cells transfected with *MK* or *tMK* gene was larger than the control cells. In nude mice injected with *MK*-transfected or *tMK*-transfected cells, visible tumor was observed earlier and the tumor tissues were larger in size and weight than in control animals that were injected with cells without the transfection of either genes.

CONCLUSION: Over-expressed *MK* or *tMK* can promote human gastric cancer cell growth *in vitro* and *in vivo*, and *tMK* has greater effect than *MK*. *tMK* may be a more promising gene therapeutic target compared with *MK* for treatment of malignant tumors.

INTRODUCTION

Midkine (*MK*), a heparin-binding growth factor, was discovered through screening for factors that mediate retinoic acid-induced cell differentiation by Kadomatsu in 1988^[1]. *MK* is a cysteine- and basic amino acid-rich protein, which is composed of two domains, i.e., N- and C-terminal half domains. The two domains are linked by a flexible linker region. Although the precise relationship between structural features and biological activities remains to be elucidated, it is interesting that only the C-terminal half domain of *MK* retains biological activities^[2,3]. *MK* gene maps to band 11p11.2^[4] and consists of five exons. Exon 1 does not encode amino acid sequence. Exon 2 encodes the hydrophobic leader sequence, which constitutes the beginning of gene translation. The signal peptide cleavage site lies toward the 3' end of exon 2^[2]. A truncated form of *MK* (*tMK*), which lacks exon 3 encoding the N-terminal half, was found in pancreatic carcinoma cell lines by Kaname in 1996^[5]. Recently two novel truncations of the *MK*, *tMKB* and *tMKC*, were found in a number of tumor cell lines, including A549 cells (lung adenocarcinoma), SGC-7901 cells (gastric cancer), 8910 cells (ovarian tumor) and MG-63 cells (osteosarcoma)^[6].

Many evidences showed that *MK* is expressed at higher levels in various tumors, such as digestive, lung, liver and breast cancers, neuroblastoma and Wilms' tumor^[7-10]. *tMK* was found in pancreatic, gastric, Wilms', colorectal, bile duct and breast tumors, but not in non-cancerous and normal tissues^[5,11-14]. *MK* can promote Wilms' tumor cell proliferation and tumor angiogenesis^[7,10,15], inhibit tumor cell apoptosis, induce transformation of NIH3T3 cells, and protect patocellular carcinoma (HCC) cells

against TRAIL-mediated apoptosis^[16-19]. *MK* and *tMK* are correlated positively with metastasis of HCC, prostate carcinomas, Lewis lung carcinoma, gastric cancer^[20-23] and gastrointestinal carcinomas^[24]. They can induce the transformation of SW-13 cells and shorten the latency of tumor formation in nude mice^[25].

Our previous study also showed that *MK* highly expressed in gastric cancer tissues of Chinese patients, and the expressions of *MK* mRNA and protein were both associated with the clinical stage and distant metastasis of gastric cancer^[26]. Therefore, it is necessary to determine the roles of *MK* and *tMK* in both tumorigenesis and tumor development in gastric cancer. BGC823 cell is a poorly differentiated gastric adenocarcinoma cell line and is an idea *in vitro* model for studying the tumorigenic activity. In the present study, we obtained human *MK* and *tMK* cDNA from gastric carcinoma tissues, constructed *MK* or *tMK* over expression plasmids (Figure 1), and then transfected the plasmids into BGC823 cell to study the effect of *MK* and *tMK* on tumorous characteristics *in vitro* and *in vivo*.

MATERIALS AND METHODS

Plasmids construction

Plasmids with *MK* and *tMK* eukaryotic expression were constructed^[12,5,13] (Figure 1). In our previous work, we designed pMD18-T-*MK* and pMD18-T-*tMK* vector^[27,28], and prepared the human *MK* and *tMK* DNA fragments by PCR using *MK-1* and *tMK* primers, (Table 1). The products of PCR digested with *Hind*III and *Eco*R I were inserted into the eukaryotic expression plasmid vector pcDNA3.1 (+) (Invitrogen, Carlsbad, CA, USA), which resulted in the formation of pcDNA3.1/*MK* and pcDNA3.1/*tMK*. The resultant recombinant plasmids were characterized by detailed restriction digestion (Figure 2).

Cell culture and transfection

BGC823, a poorly differentiated gastric adenocarcinoma cell line, was cultured in RPMI medium 1640 (Gibco/BRL) supplemented with 10% fetal calf serum (Si Ji Qing, China) at 37°C under 5% humidified CO₂ and 100 µg/mL each of streptomycin and penicillin G (Amresco, USA). The plasmid was transfected using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Briefly, approximately 0.8×10^5 cells/well were grown overnight in 24-well plates. When the cells reached 90%-95% confluence, they were transfected with 0.8 µg of pcDNA3.1/*MK* or pcDNA3.1/*tMK* or pcDNA3.1 in serum-free medium using Lipofectamine 2000. After 4 h incubation at 37°C, 400 µL RPMI 1640 with 10% FBS was added. Stable transfectants were selected in the presence of 400 mg/L G418 (Amersco) during 2 wk of culture.

RNA extraction and RT-PCR

Total RNA was extracted using the TaKaRa RNAiso Reagent (TaKaRa, Japan) according to the manufacturer's instructions. RNA concentrations were quantified by spectrophotometer at 260 nm. One µg total RNA was reverse-transcribed using Revert Aid™ First Strand cDNA Synthesis Kit (Fermentas, USA). Subsequently, 2 µL

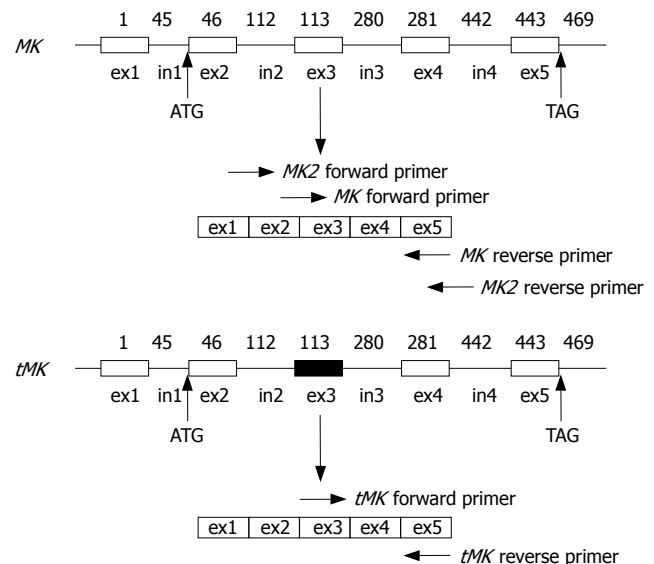


Figure 1 Illustration of *MK* and *tMK* gene DNA structures. Box: Exon (ex); Line: Intron (in); Shaded box: Truncated portion; ATG: Start site; TAG: Terminal site; Numeric figures: Nucleotide position of the mRNA transcript. Arrowheads indicate the sites of primer complemented with *MK* or *tMK* mRNA.

of the incubation mixture was used as the template for the following PCR using 2 × Taq enzyme mix kit (Tian Gen, China). Primers were synthesized by Bioasia (Shanghai, China) and are listed in Table 1. PCR was carried out for 28 or 30 cycles of denaturation (30 s at 94°C), annealing (40 s at 55°C), and extension (30 s at 72°C). The PCR products were then detected on 1% agarose gel containing 0.5 mg/L ethidium bromide. The gel was put on an UV-transilluminator and photographed. The *MK* signal was measured by a densitometer and standardized against the β-actin signal using a digital imaging and analysis system (SmartSpec™ Plus, BIO-RAD, USA).

Western blot analysis

Cells (1×10^7) were lysed in a buffer containing 50 mmol/L Tris-Cl, pH8.0, 150 mmol/L NaCl, 0.02% NaN₃, 0.1% SDS, 100 mg/L phenylmethylsulfonyl fluoride (PMSF) and 1 mg/L Aprotinin, 1% Triton. After centrifugation, cell lysates (75 µg/lane) were subjected to 15% SDS-PAGE and transferred onto polyvinylidene difluoride membranes (Millipore, USA). The membranes were blocked for 1 h in PBST (10 mmol/L Tris-HCl, pH 7.4, 150 mmol/L NaCl, 0.05% Tween-20) containing 2% nonfat dried milk. Antibodies specific for *MK* (1:400, BA1263, Boster, China), β-actin (1:400, BA0410, Boster) and HRP-conjugated goat anti-rabbit secondary antibody (1:2000, BA1054, Boster) were used. Protein bands were detected by the enhanced chemiluminescence (ECL) reaction (Kibbutz Beit Haemek, Israel).

Proliferation analysis

Cell viability was assessed with a Cell Counting Kit (Dojin Laboratories, Kumamoto, Japan). Briefly, BGC823 cells transfected with pcDNA3.1/*MK*, pcDNA3.1/*tMK*, or pcDNA3.1 and parental BGC823 cells were plated onto 96-well plates in RPMI 1640 supplemented with 10% FBS at a density of 3×10^3 cells/well. After 4 h, the medium

Table 1 Primers used in this study

Primers	Sequence 5'-3'	Reference	Expected size (bp)	Cycles of PCR
MK-1 sense	AAAAAAGCTTATGAAAAAGAAAGATAAGGTGAAGAAG		389	28
MK-1 antisense	AAAAGAATTCCTAGTCCTTCCCTTCCCT			
tMK sense	AAAAAAGCTTATGAAAAAGAAAGCCGACTG	Paul <i>et al</i> , 2001 ^[13]	221	28
tMK antisense	AAAAGAATTCCTAGTCCTTCCCTTCCCT			
MK-2 sense	ATGCAGCACCGAGGCTTCCT	Kaname <i>et al</i> , 1996 ^[5]	447	30
MK-2 antisense	ATCCAGGCTTGGCGTCTAGT		279	
β-actin sense	CCACGAACTACCTTCAACTC		270	28
β-actin antisense	TCATACTCCTGCTGCTTGCTGATCC			

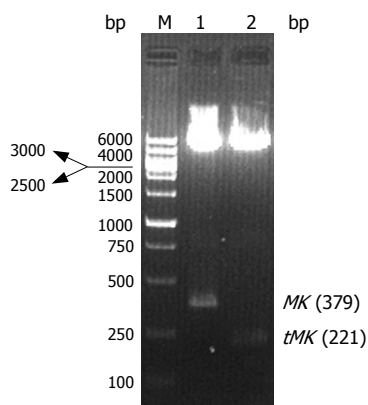


Figure 2 Restriction digestions of recombinant plasmids. M: Wide range DNA marker 100-6000 (TaKaRa); Lane 1: pcDNA3.1/MK; Lane 2: pcDNA3.1/tMK.

was changed to serum-free medium, and the cells were cultured ≤ 2 d. Ten microliter of a solution containing 4-[3-(2-methoxy-4-nitrophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate sodium salt (WST-8) was added to each well. Following incubation of an additional 4 h, the absorbance was measured at 450 nm with a multi-detection microplate reader (HynergyTM HT, BIO-TEK, USA).

Colony formation in soft agar

To perform the soft agar assay, a base layer of 0.5% (w/v) agar was prepared by adding autoclaved 1% (w/v) agar solution to 2x RPMI-1640 supplemented with 20% fetal calf serum at a 1:1 ratio. Stable transfectants or parental cell suspension containing 2×10^5 cells were prepared in a 1:1 mixture of 0.7% (w/v) agar solution and 2x RPMI-1640 supplemented with 20% FCS. Cell suspension was added to the top of the base layer, allowed to solidify, and the plate was incubated at 37°C in a humidified 5% CO₂. The plates were incubated for 10-15 d. The number of colonies was determined by direct counting under microscopy. Counts were expressed as number of colonies per plate on average from three independent experiments.

Wound healing assay

The transfected BGC823 cells with pcDNA3.1/MK, pcDNA3.1/tMK or pcDNA3.1 and parental cells were plated onto 6-well plates in RPMI 1640 supplemented with 10% FBS at a density of 2×10^5 cells/well. After 4 h, the medium was changed to serum-free medium. After 24 h, a plastic cell scraper was used to make an approximate 0.6 mm gap on the cell monolayer. Migration was quantitated by determining the distance between the cell edges at 0, 24 h and 48 h at the four marked locations on each well, using

an inverted microscope with a scale in the eyepiece^[29]. The results of the four readings from each well were averaged. Experiments were repeated three times.

Tumorigenicity study in vivo

Female BALB/c nude mice (5-6 wk old) were obtained from Vital River Lab Animal Co, Ltd, Beijing Laboratory Animal Research Center (Beijing, China). Cultured cells were harvested by trypsinization, washed and suspended in PBS at 10^7 cells/mL. One hundred μ L cell suspensions were injected subcutaneously into the flank of female nude mice (seven mice per cell line). Tumor diameters were measured on d 14, 21 and 28, and tumor volume in mm³ was calculated by the formula: Volume = (width)² \times length/2. Tumor growth rates were calculated by the formula: TGR = (V_{28th} - V_{21th})/7 d. Data were presented as mean \pm SE. Twenty-eight days after injection, nude mice were sacrificed, and the tumors were removed, photographed and weighed.

Immunohistochemistry

Immunostaining was performed on 6- μ m tissue sections using strept-avidin-biotin staining kit (Boster). For antigen retrieval, slides were heated by microwave in 0.01 mol/L Tri-sodium citrate buffer. Nonspecific binding sites were blocked with 5% BSA for 30 min and endogenous peroxidase activity was suppressed by treatment with 3% H₂O₂ in methanol for 30 min. Sections were exposed to rabbit polyclonal anti-MK antibody (1:250, Boster) overnight at 4°C. 3,3-diamino-enzidine was used as chromogen (Boster). Counterstaining was done with hematoxylin. Negative control sections were incubated with PBS instead of anti-MK antibodies. In each step, samples were washed with PBS.

Statistical analysis

Results were presented as mean \pm SE. Statistical significance between groups was analyzed by one-way ANOVA followed with the Student-Newman-Keuls multiple comparison tests. A *P* value of < 0.05 was considered significant. Frequency of tumorigenesis in nude mice was calculated by Fisher's exact test.

RESULTS

Expression of MK and tMK

To evaluate the roles of MK and tMK in gastric tumorigenesis, we used transfection assay to obtain a

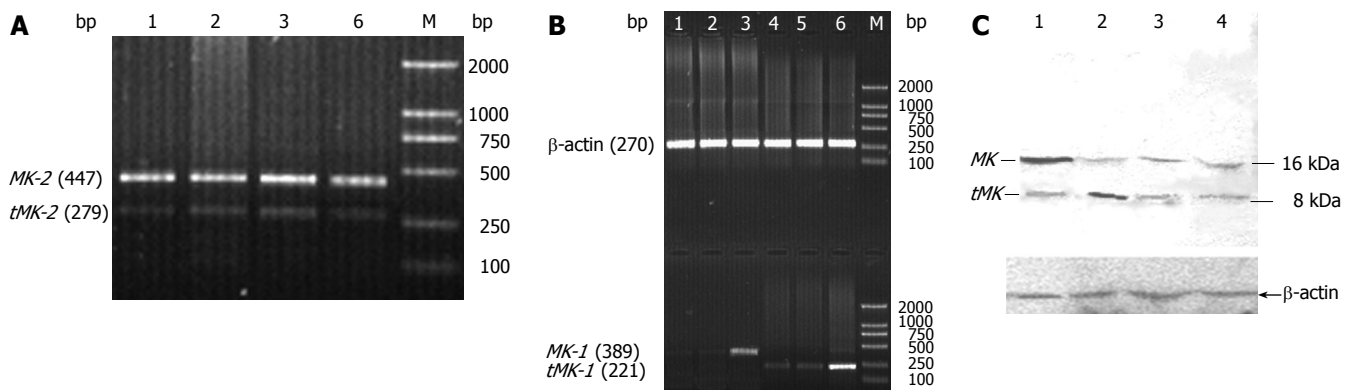


Figure 3 RT-PCR (A, B) and Western blotting (C) analysis of the expression of *MK* or *tMK* in BGC823 after transfection. A and B, M: DNA molecular weight standards, DL2000 (TaKaRa); Lane 1 and 4: BGC823; Lane 2 and 5: BGC823/vector; Lane 3: BGC823/*MK*; Lane 6: BGC823/*tMK*. C, Lane 1: BGC823/*MK*; Lane 2: BGC823/*tMK*; Lane 3: BGC823/vector; Lane 4: BGC823.

MK or *tMK* over-expressed gastric cell line. RT-PCR and Western blotting were performed to determine *MK* or *tMK* expression level in the transfected gastric carcinoma cells. Compared with the parental cells and pcDNA3.1 transfected cells, transfection of BGC823 cells with pcDNA3.1/*MK* or pcDNA3.1/*tMK* resulted in significant enhancement of *MK* or *tMK* expression in BGC823 cells. These results indicated that transfection of pcDNA3.1/*MK* and pcDNA3.1/*tMK* was successful (Figure 3B and C).

Effect of over-expression of *MK* or *tMK* on BGC823 cells

To determine whether over-expression of *MK* and *tMK* could affect the BGC823 cell growth, cell proliferation activity was detected using Cell Counting Kit. The transfection of pcDNA3.1/*MK* or pcDNA3.1/*tMK* to BGC823 significantly increased the proliferation of BGC823 cells compared with the control. This showed that over-expressed *MK* or *tMK* could accelerate the cellular proliferation at 12 h, 24 h, 36 h and 48 h. Moreover, *tMK* exhibited stronger stimulatory effect than *MK* (Figure 4A). No difference between BGC823/vector and BGC823 was detected (Figure 4A). Furthermore, colony-forming assay was conducted in BGC823, BGC823/vector, BGC823/*MK* and BGC823/*tMK* (Figure 4B and C). The results showed that the colony number of BGC823/*MK* and BGC823/*tMK* cells was increased by 2- to 3-fold compared with BGC823 and BGC823/vector (Figure 4C). In addition, the wound healing assay also showed that over-expressed *MK* or *tMK* could induce significant migration of the cell at 24 h and 48 h, about 1.5-fold over BGC823 and BGC823/vector cells, and *tMK* showed stronger effect than *MK* (Figure 4D). These results demonstrated that over-expression of *MK* and *tMK* significantly enhanced the malignant state and invasive ability of BGC823 cells.

Tumor growth promoted by *MK* or *tMK* in vivo

As the over-expression of *MK* or *tMK* significantly changed the behavior of BGC823 cells *in vitro*, it is necessary to analyze the tumorigenicity of the stable transfectant *in vivo*. The time and frequency of visible tumor in nude mice treated with BGC823, BGC823/vector, BGC823/*MK* and BGC823/*tMK*, respectively, are

presented in Table 2. Tumor was clearly observed in most BGC823/*MK*- and all BGC823/*tMK*-injected mice at d 7, whereas visible tumor formed in about half of BGC823/vector and BGC823 injected mice until d 14. Furthermore, tumor diameters and volume were subsequently measured at d 14, 21 and 28. The results showed that tumor volumes of mice injected with BGC823/*MK* or BGC823/*tMK* cells were significantly larger than the control at d 21 and 28 (Figure 5C). Tumor growth rate (TGR) from d 21 to 28 showed that the TGR of nude mice injected with BGC823/*MK* or BGC823/*tMK* was significantly higher than the control mice (Figure 5D). At d 28 after inoculation, the tumors were removed, photographed and weighed. The tumor in mice injected with BGC823/*MK* and BGC823/*tMK* cells was 2-fold of that of the control (Figure 5B), and tumors in two mice injected with BGC823/*tMK* cells had erosive appearance (Figure 5A). Apparently, BGC823/*MK* or BGC823/*tMK* transfected cells could multiply and grow earlier and more rapidly than the BGC823 and BGC823/vector control cells in nude mice.

Immunohistochemical analysis

To detect whether BGC823/*MK*- or BGC823/*tMK*-transfected cells can stably express *MK* or *tMK* in nude mice for an extended period and the association between tumor growth and *MK* or *tMK* protein levels, immunohistochemical staining was conducted. *MK* was detected in cytoplasm and nucleus of tumor cells from different treatment groups of mice. The number and density of the positive points in tumor tissues induced with BGC823/*MK* and BGC823/*tMK* cells were evidently higher than the cells treated with BGC823 and BGC823/vector (Figure 6).

DISCUSSION

To determine whether *MK* and *tMK* contribute to gastric tumorigenesis and tumor development, BGC823 cells that over-expressed *MK* and *tMK* genes, and nude mice inoculated with the BGC823 cells over-expressing either *MK* or *tMK* were used as model systems *in vitro* and *in vivo*, respectively. To show that the upregulated *MK* and *tMK*

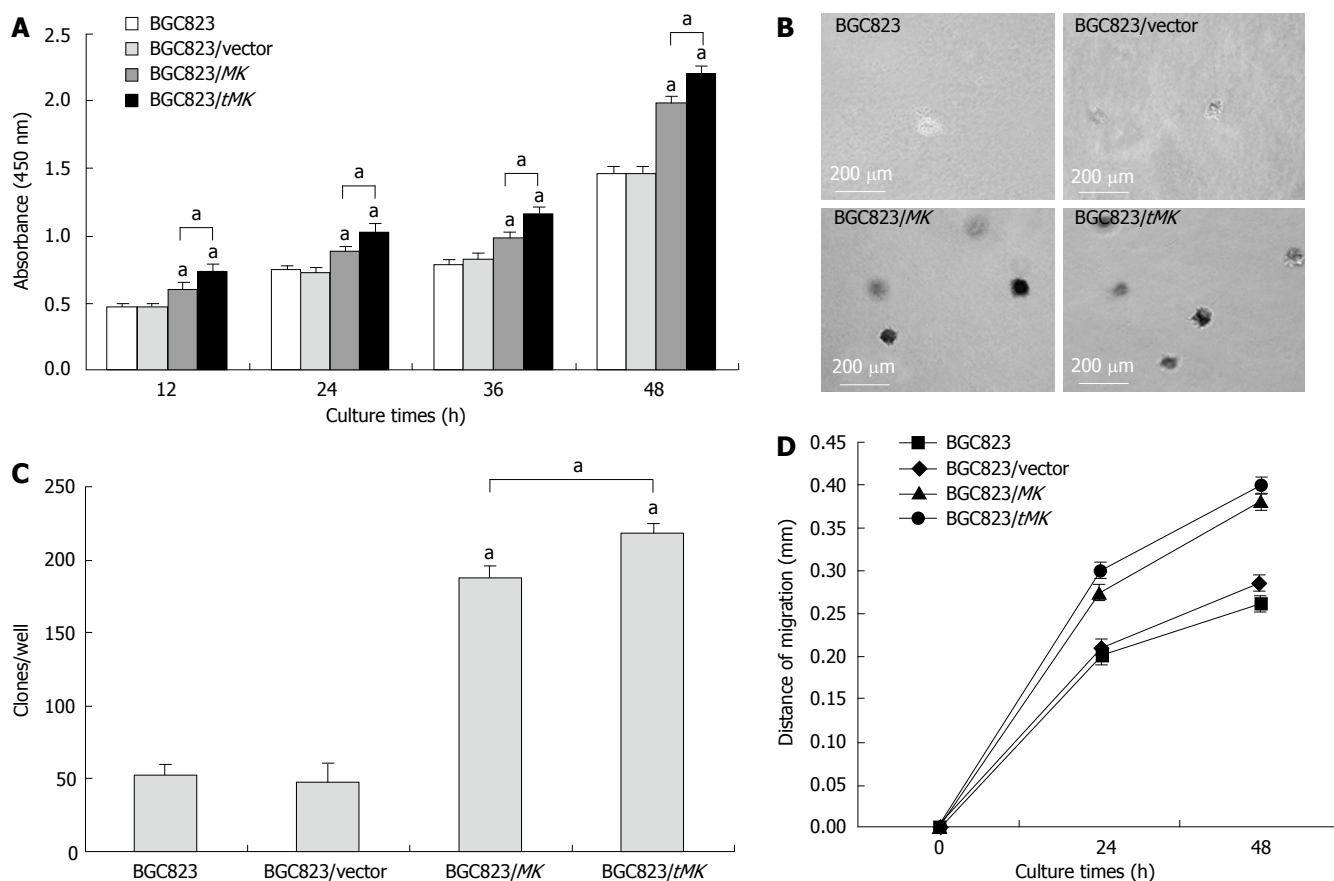


Figure 4 Effects of over-expressed *MK* or *tMK* on BGC823 cells *in vitro*. **A**: The cell proliferation determined by Cell Counting Kit ($P < 0.05$); **B**: Colony formation in soft agar observed under light microscope; **C**: Comparison of colony numbers. **D**: Analysis of cell migration.

were exogenous in the transfected cells, we designed another pair of primers for *MK*-2 sequence (Table 1)^[5]. The forward primer of *MK*-2 was complemented with the start section of exon 2, and the reverse primer was complemented with exon 5 and several base pairs of 3'-untranslated regions. *tMK* lacks exon 3, so *MK* (448 bp) and *tMK* (296 bp) DNA were obtained at the same time by RT-PCR using primers for *MK*-2. There was no significant difference in the expression of *MK* and *tMK* between transfected cells and parental cells. The state in those cells transfected with or without *MK* and *tMK* genes can imitate *MK* and *tMK* expression from initial to metastatic stages of tumor formation.

Previous studies showed that the over-expression of *MK* in S462 cell (malignant peripheral nerve sheath tumor cell line) could increase the cell viability and protect the cells from apoptosis under serum deprivation, but did not induce the proliferation of S462 cells to promote xenograft tumor growth in nude mice^[16]. *MK* and *tMK* can induce the transformation of SW-13 cells (adrenal carcinoma cell line) and shorten the latency of tumor formation in nude mice, but SW-13/*MK* and SW-13/*tMK* showed no difference in tumor growth rate from the control^[25]. However in our study, the growth of BGC823 cells which over-expressed *MK* and *tMK*, was increased significantly compared with the control cells. The tumor formation time was shortened in nude mice injected with BGC823/*tMK* or BGC823/*MK* cells. Tumor growth rate of was significantly higher than the control, and tumor volume and weight were higher than the control, indicating that the idiographic effect of

Table 2 Frequency of tumorigenesis in nude mice

Injected cells	No. of mice	No. of days to tumor detection (percent of tumorigenesis)			
		7	14	21	28
BGC823	7	0 (0.00)	3 (42.86)	6 (85.71)	7 (100)
BGC823/vector	7	0 (0.00)	4 (57.14)	7 (100)	
BGC823/ <i>MK</i>	7	5 (71.43) ^a	6 (85.71) ^a	7 (100)	
BGC823/ <i>tMK</i>	7	7 (100) ^b			

P value was calculated by Fisher's exact test. 7 d: BGC823/*MK* vs BGC823 or BGC823/vector, $P = 0.0105$; BGC823/*tMK* vs BGC823 or BGC823/vector, $P = 0.0003$. 14 d: BGC823/*MK* vs BGC823, $P = 0.0174$; BGC823/*MK* vs BGC823/vector, $P = 0.0489$. ^a $P < 0.05$; ^b $P < 0.01$.

MK and *tMK* on tumorigenesis and tumor development may be related to types of tumors.

MK and *tMK* are heparin-binding growth factors. They play fundamental roles in the regulation of cell differentiation and development. Their aberrant expressions are usually associated with tumorigenesis^[30-33]. In our study, *tMK*, which was only found in cancer tissues, had stronger effects than *MK* on tumor cell proliferation, and tumors from two mice injected with BGC823/*tMK* cells had erosive appearance. This result was in agreement with the previous studies. The differential activities of *MK* and *tMK* in promoting tumor proliferation may be attributed to the difference of the tertiary structure between these two proteins^[34].

In conclusion, over-expressed *MK* or *tMK* could promote tumor development of human gastric cancer

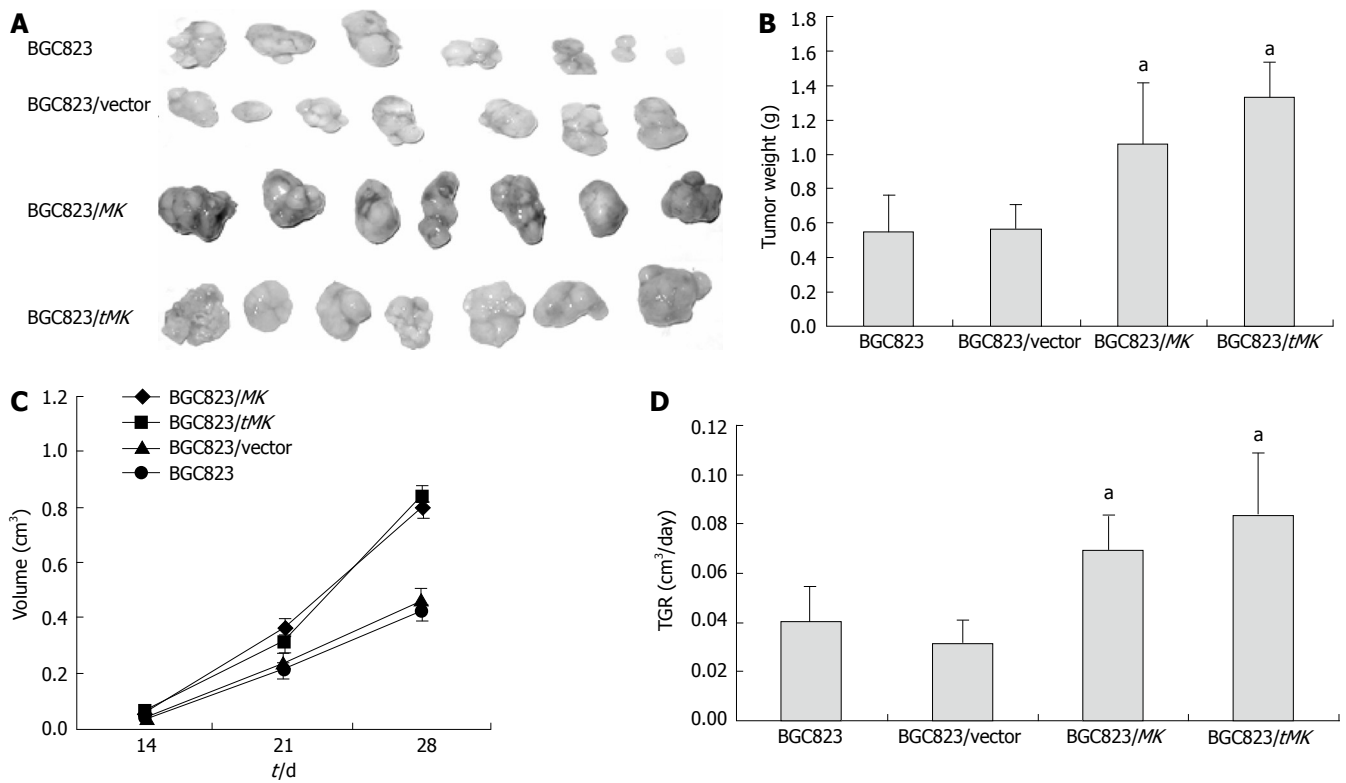


Figure 5 Promotion of tumorigenesis of *MK*- or *tMK*-transfected cells *in vivo*. **A**: Photograph of tumor size; **B**: Comparison of tumor weight ($P < 0.05$); **C**: Measure of tumor volume ($^aP < 0.05$); **D**: Analysis of tumor growth rate ($^aP < 0.05$).

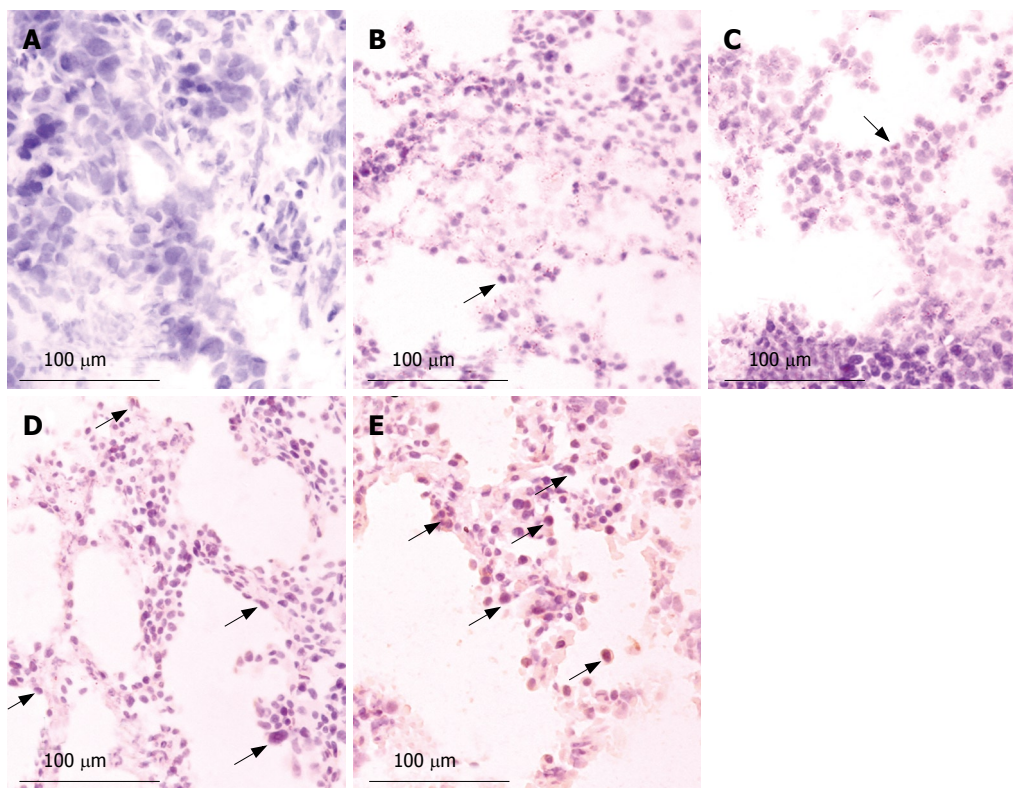


Figure 6 Immunohistochemical staining of tissues for *MK* and *tMK* with rabbit polyclonal anti-*MK* antibody. **A**: Negative control sections; **B**: Tumor tissue from BGC823 injected mice; **C**: Tumor tissue from BGC823/vector injected mice; **D**: Tumor tissue from BGC823/*MK* injected mice; **E**: Tumor tissue from BGC823/*tMK* injected mice ($\times 200$). Arrows represent positive results of *MK* or *tMK* expressions.

and tumorigenesis *in vitro* and *in vivo*. *tMK* had greater effect than *MK* in promoting the tumor formation. *tMK* might become a more promising gene therapeutic target compared with *MK* for treatment of tumors.

COMMENTS

Background

Midkine (*MK*), a heparin-binding growth factor, and its truncated form (*tMK*), were found expressing at higher levels in various tumors, and involve the growth and metastasis of some carcinomas. The expressions of *MK* mRNA and the protein

are both associated with the clinical stage and distant metastasis of gastric cancer in the Chinese patients. But few studies were conducted on the roles of *MK* and *tMK* in both tumorigenesis and tumor development in gastric cancer. In this article, the effect of *MK* and *tMK* on the growth and metastasis of BGC823 (a poorly differentiated gastric adenocarcinoma cell line), and tumorigenesis in nude mice was investigated.

Research frontiers

Many studies of *MK* and *tMK* expression in various tumors including gastric cancer, have been reported. It has been found that *MK* can promote Wilms' tumor cell proliferation and tumor angiogenesis, inhibit tumor cell apoptosis, induce transformation of NIH3T3 cells, and protect hepatocellular carcinoma cells against TRAIL-mediated apoptosis. However, there has been no investigation about the effect of *MK* and *tMK* on the characteristics of gastric carcinoma.

Innovations and breakthroughs

This article suggests that over-expressed *MK* and *tMK* can promote BGC823 cell growth, colony formation, wound healing and tumorigenesis in nude mice. *tMK* had greater effect than *MK*, and it might become a promising gene therapeutic target for treatment of malignant tumors.

Applications

This observation might be of potential value in gene therapy for gastric cancer.

Peer review

The manuscript describes that over-expressed *MK* and *tMK* can promote BGC823 cell growth, colony formation, wound healing and tumorigenesis in nude mice. The results were found important for *MK* and *tMK* as gene therapeutic target in gastric cancer.

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CLINICAL RESEARCH

Clinical and endoscopic features of Chinese reflux esophagitis patients

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Abstract

AIM: To analyze the clinical and endoscopic features of Chinese patients with reflux esophagitis (RE).

METHODS: A total of 1405 RE patients were analyzed retrospectively. Data on gender, age, presence/absence of *H pylori* infection and associated esophageal hiatal hernia were collected. Esophagitis was divided into different grades according to Los Angeles Classification.

RESULTS: Of 18823 patients, 1405 were diagnosed as RE. The ratio of male to female patients was 1.75:1 ($P < 0.01$). The mean age of male and female patients was significantly different ($P = 0.01$). The peak age at onset of the disease was 40-60 years. According to Los Angeles Classification, there were significant differences in the age of patients with grades A and B compared to patients with grades C and D ($P < 0.01$). Two hundred and seventy-seven patients were infected with *H pylori*, the infection rate was low ($P < 0.01$). Complication of esophageal hiatal hernia was found to be significantly associated with the severity of esophagitis and age in 195 patients ($P < 0.01$). Esophageal mucosa damages were mainly located at the right esophageal wall.

CONCLUSION: The peak age of onset of RE is 40-60 years and higher in males than in females. The mean age of onset of RE is lower in males than in females. The infection rate of *H pylori* is significantly decreased in patients with esophagitis. Old age and esophageal hiatal hernia are associated with more severe esophagitis. Right esophageal mucosal damage can occur more often in RE patients.

INTRODUCTION

Gastroesophageal reflux disease (GERD) is described as a chronic symptom and/or tissue damage caused by abnormal gastric content refluxing up into the esophagus. GERD is a common disease, with associated typical symptoms of heartburn and regurgitation^[1]. In recent years, the questionnaire survey among 5000 subjects in Beijing and Shanghai revealed that 10.19% and 7.76% of the subjects have associated reflux symptoms and it is, thus, speculated that GERD has a prevalence of 5.77%^[2]. A community-based investigation in Guangdong Province showed that heartburn and/or regurgitation occurs at least one week in 6.2% of the community population^[3]. It was reported that the prevalence of GERD varies greatly, from 7% to 15% and even to over 20%^[4]. Patients with GERD have manifestations of esophageal mucosal damages, such as reflux esophagitis (RE), non-erosive GERD (reflux disease) or negative endoscopy reflux disease (NERD)^[5-7]. As a common gastrointestinal disease, RE has attracted widespread attention at home and abroad. To further understand RE and summarize its clinical and endoscopic features, a total of 1405 patients with RE undergoing endoscopic examination in the Digestive Endoscopy Center of our hospital were retrospectively analyzed in the present study.

MATERIALS AND METHODS

Ascertainment methods

A total of 18823 patients underwent endoscopic

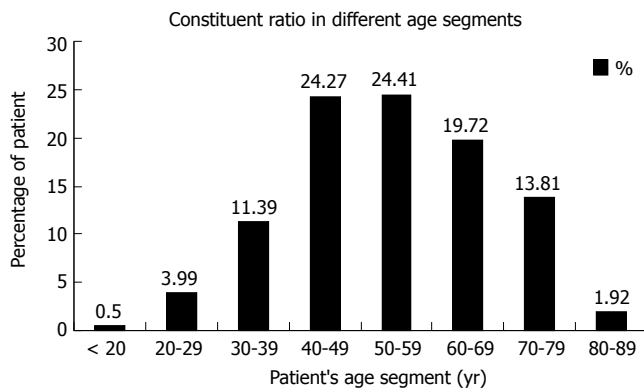


Figure 1 Percentage of patients at different age stages.

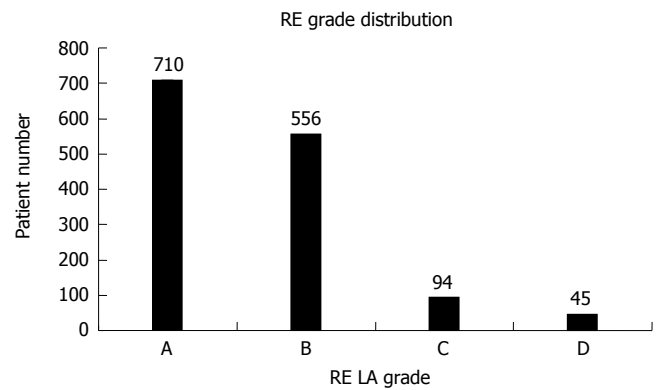


Figure 2 Distribution of disease grades in patients with reflux esophagitis.

examinations in the Digestive Endoscopy Center of Beijing Friendship Hospital between September 2004 and January 2007. The unified questionnaire was established and carefully filled in by specialists mainly based on endoscopic diagnosis. The general items included in the questionnaire for RE cases were gender, age, *etc.* The diagnostic data about the patients with RE included endoscopic staging, presence of associated *H. pylori* infection and esophageal hiatal hernia (EHH), *etc.*

General data

A total of 18823 patients (9800 males and 9023 females) underwent endoscopic examinations in the Digestive Endoscopy Center of Beijing Friendship Hospital between September 2004 and January 2007. Of them, 1405 patients (895 males and 510 females) were diagnosed as reflux esophagitis. Their age was 15- 89 years.

Diagnostic criteria

Esophagitis was divided into different grades according to Los Angeles Classification in patients with RE. Patients with upper gastrointestinal operation-induced lumen structural changes, upper gastrointestinal obstruction, esophageal varices, achalasia of cardia, and patients undergoing esophageal stenting and those with combined reflux esophagitis following three-cavity catheter or gastric tube implantation were excluded.

H. pylori infection was evaluated based on the diagnosis by rapid urease staining, C¹³ breath test or pathological silver staining. Esophageal hiatal hernia was diagnosed when dentate line shifted 2 cm or more upward under endoscope, and hernia sac was seen under intra-gastric reversal endoscope. Mucosa within the hernia sac was diagnosed as gastric mucosa. Furthermore, esophageal hiatal hernia could be definitely diagnosed according to the upper gastrointestinal contrast.

A 1:00-12:00 location mark of esophageal mucosa damage similar to the index dial was established by setting the midpoints of anterior, posterior, left and right esophageal walls as 12:00, 6:00, 9:00 and 3:00, respectively. The location of mucosal damage was expressed as the corresponding location mark.

Statistical analysis

$P < 0.05$ was considered statistically significant.

Table 1 Comparison of age in patients with different grades of reflux esophagitis

	LA-A	LA-B	LA-C	LA-D
Mean age	53.35 ^b	54.53 ^b	60.50	61.44
SD	13.90	14.19	13.68	14.97

^b $P < 0.01$ vs the age of patients with grades C and D of RE. LA-A: Los Angeles Classification grade A; LA-B: Los Angeles Classification grade B; LA-C: Los Angeles Classification grade C; LA-D: Los Angeles Classification grade D.

RESULTS

Reflux esophagitis, age and grade

A total of 1405 patients were diagnosed as reflux esophagitis, accounting for 7.46% of the 18823 patients undergoing gastroscopic examinations. The diagnosis rate was 9.13% in 895 male patients and 5.65% in 510 female patients. The ratio of male to female patients was 1.75:1 ($P < 0.01$). The age of onset of RE was 15-89 years (mean age: 54.56 ± 14.19 years). The mean age of male and female patients was 53.82 ± 14.19 years and 55.85 ± 14.08 years, respectively ($P = 0.01$). From the age of 20 to 90 years, 10 years were identified as one age stage. The number and percentage of patients in each stage were 56 and 3.99%, 160 and 11.39%, 341 and 24.27%, 343 and 24.41%, 277 and 19.72%, 194 and 13.81%, and 27 and 1.92%, respectively. There were 7 patients at the age of less than 20 years, accounting for 0.5% (Figure 1). According to Los Angeles Classification, there were 710 patients with grade A (mean age 53.35 ± 13.90 years), 556 with grade B (54.53 ± 14.19 years), 94 with grade C (60.50 ± 13.68 years) and 45 with grade D (61.44 ± 14.97 , Figure 2). Patients with grades A and B accounted for 90.1% of all the patients. There was no difference in the age of patients with grades A and B ($P = 0.138$) or with grades C and D ($P = 0.712$). However, there were significant differences in the age of patients with grades A and B compared with those with grades C and D ($P < 0.01$, Table 1).

Reflux esophagitis and *H. pylori* infection

Of the 18823 patients undergoing endoscopic examination, 7190 were infected with *H. pylori*, the infection rate was 38.2%. Of the 1405 patients with reflux esophagitis, 277 were infected with *H. pylori*, the infection rate was 19.7%,

Table 2 Relationship between reflux esophagitis and *H pylori* infection

	Patients (n)	RE patients (n)	Male	Female	LA-A	LA-B	LA-C	LA-D
<i>H pylori</i> +	7190	277	188	89	137	116	15	9
<i>H pylori</i> -	11633	1128	707	421	576	440	79	36
Percentage (%)	38.2	19.7 ^b	21.01	17.45	19.3	20.86	15.96	20
Total	18823	1405	895	510	710	556	94	45

^b $P < 0.01$ vs *H pylori* infection rate in all patients undergoing endoscopic examination. RE: Reflux esophagitis; LA-A: Los Angeles Classification grade A; LA-B: Los Angeles Classification grade B; LA-C: Los Angeles Classification grade C; LA-D: Los Angeles Classification grade D; *H pylori*+: Infected with *H pylori*; *H pylori* -: Not infected with *H pylori*.

Table 3 Relationship of reflux esophagitis and esophageal hiatal hernia

	RE patients (n)	Mean age	Male	Female	LA-A	LA-B	LA-C	LA-D
EHH+	195	62.03 ± 14.11	122	73	48	74	48	25
EHH-	1210	53.35 ± 13.83 ^b	773	437	662	482	46	20
Percentage (%)	13.9		13.63	14.31	6.76 ^a	13.31 ^a	51.06 ^a	55.56 ^a
Total	1405	54.56 ± 14.19	895	510	710	556	94	45

^b $P < 0.01$ vs the age of patients with esophageal hiatal hernia; ^a $P = 0.717$ (no difference in the detection rate of grades C and D of RE; $P < 0.01$ (significant differences in the detection rate of esophageal hiatal hernia among the other patients; EHH+: Associated esophageal hiatal hernia; EHH-: No esophageal hiatal hernia).

which was significantly lower than that (38.2%) of all patients undergoing endoscopic examinations during the same period. Of the 277 patients infected with *H pylori*, 188 were males and 89 were females. There was no gender difference in *H pylori*- infected patients with esophagitis ($P = 0.109$). Of the 277 *H pylori*- infected patients with esophagitis, 137 had grade A, 116 had grade B, 15 had grade C and 9 had grade D, respectively. The severity of esophagitis was not associated with *H pylori* infection ($P = 0.71$, Table 2).

Reflux esophagitis and esophageal hiatal hernia

Of the 1405 patients with reflux esophagitis, 195 had esophageal hiatal hernia (EHH+), accounting for 13.9%. Their mean age was 62.03 ± 14.11 years. There was a significant difference in the age between patients with and without esophageal hiatal hernia ($P < 0.01$). No statistical significance was found in 122 male and 73 female patients ($P = 0.722$). Of the 195 patients with esophageal hiatal hernia, 29 were infected with *H pylori*. The occurrence of esophageal hiatal hernia was not associated with the presence of *H pylori* infection ($P = 0.08$). In the 195 patients with esophagitis and esophageal hiatal hernia, 48 had grade A, 74 had grade B, 48 had grade C and 25 had grade D, respectively. There was no difference in the detection rate of esophageal hiatal hernia between patients with grades C and D ($P = 0.717$), while there were significant differences in the detection rate of esophageal hiatal hernia among the other patients ($P < 0.01$, Table 3).

Esophageal mucosal damage in reflux esophagitis patients

In the present study, the number and location of esophageal mucosal damages in patients with grades A and B reflux esophagitis were analyzed. In the 710 patients with grade A, 453 had only a mucosal damage, 184 had 2 damages, 60 had 3 damages and 13 had 4 damages (Figure 3A and B). In the

556 patients with grade B, 115 had only a mucosal damage, 230 had 2 damages, 137 had 3 damages, 67 had 4 damages, and 7 had 5 or more damages (Figure 3), indicating that a mucosal damage occurred mainly in patients with grade A and two or more mucosal damages occurred mainly in patients with grade B.

A 1:00-12:00 location mark similar to the index dial was established by setting the midpoints of anterior, posterior, left and right esophageal walls as 12:00, 6:00, 9:00 and 3:00, respectively. The location of mucosal damage was expressed as the corresponding location mark. The number of mucosal damages at the corresponding location of 1:00-12:00 was 184, 385, 288, 301, 185, 258, 212, 88, 96, 91, 97 and 157, respectively (Figure 4). The esophageal mucosa damages were mainly located at the right esophageal wall.

DISCUSSION

Population-based studies suggest that GERD is a common condition with a prevalence of 10%-20% in Western Europe and North America^[8,9]. The prevalence rates in South America (10%) and Turkey (11.9%) are similar to those in European countries^[10,11]. In Asia, the prevalence is generally lower^[12]. Chen *et al*^[3] reported that the prevalence of heartburn occurring weekly is 6.2% while Wong *et al*^[13] have found a lower prevalence of 2.3%. With the deepening of studies and understanding of gastroesophageal reflux disease, the number of such patients is increased in clinical practice. The significantly decreased quality of life in RE patients has increasingly attracted extensive attention^[14-16]. It was reported that the quality of life deteriorates as the severity of GERD increases^[17]. In the present study, all the patients undergoing endoscopic examinations in our hospital between September 2004 and January 2007 were analyzed. The detection rate of reflux esophagitis was 7.46%, which

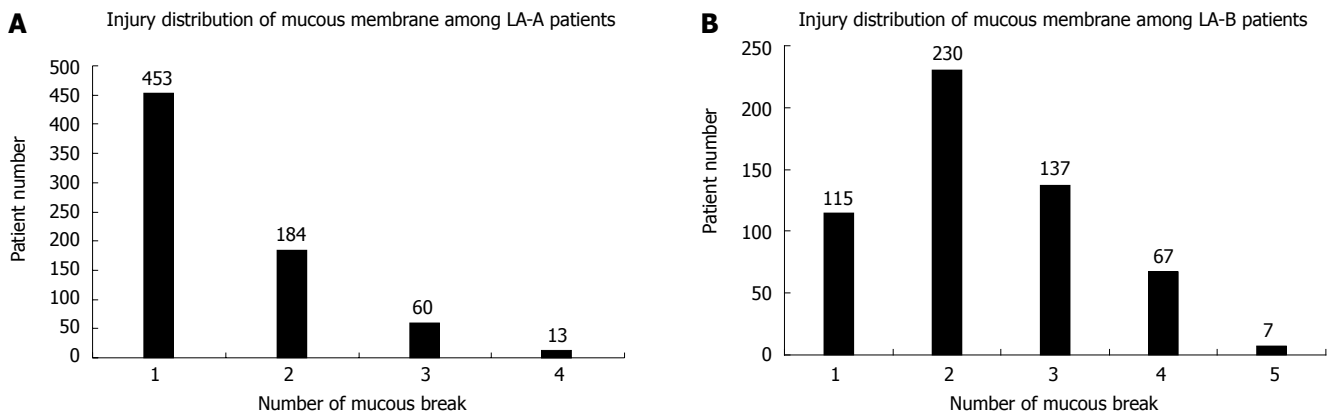


Figure 3 Distribution of mucosal damage in patients with Los Angeles Classification grade A (A) and grade B (B).

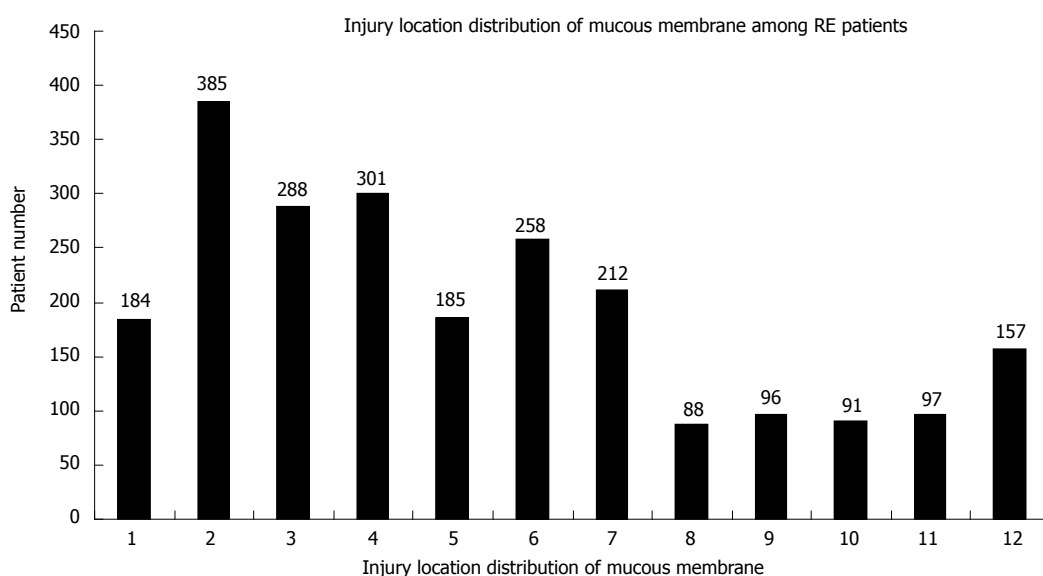


Figure 4 Distribution of location of mucosal damage in patients with reflux esophagitis.

was higher in males than in females. The ratio of male to female was 1.75:1, suggesting that males have a higher susceptibility to RE than females. In comparison to the Italian general population, the prevalence of over-weight and obesity is increased in female RE patients but not in male RE patients^[18]. Furthermore, RE tends to occur at a younger age of male patients, which may be related to the differences in life style between males and females. A study demonstrated that the prevalence increases linearly with age among women, and peaks among men at the age of 50-70 years and thereafter declines^[19]. In the present study, the patients with grades A and B of RE accounted for 90.1%, suggesting that the disease is mild in most patients. Although reflux esophagitis can occur at all age stages, most patients are 40-60 years old. Esophagitis aggravates with the age of patients with reflux esophagitis. In this study, no statistical difference was found in items such as onset age and gender between patients with grades A and B of RE. Endoscopic examination is difficult to assess the length of esophageal mucosal damage. The esophageal mucosal damage in patients with grades A and B of RE was extended along the long axis of esophagus, which was different from transversal and vertical extension of damages in patients with grades C and D of RE.

These results suggest that grades A and B of RE can be considered a same grade. According to the standards set at Yantai Meeting, reflux esophagitis can be divided into grade 0 = normal mucosa (histological changes may be observed), grade 1 = punctiform or strip redness and erosion without integration, grade 2 = punctiform or strip redness and erosion with integration but non-entire pattern, grade 3 = extensive lesions, redness, erosion integration with entire pattern or ulcers. Grade 1 is equivalent to grades A and B in Los Angeles Classification. It was reported that changes in esophageal motility and response to PPI therapy are similar between patients with grades A and B of RE^[20,21]. Therefore, we believe that the standards set at Yantai Meeting are more practical.

In the present study, the *H. pylori* infection rate was significantly decreased in patients with reflux esophagitis. *H. pylori* infection had no clear relationship with gender, age and severity of reflux esophagitis. There is evidence that *H. pylori* infection is not associated with gastroesophageal reflux disease and *H. pylori*-related inflammation does not affect sphincter motility, namely *H. pylori*-positive patients have normal LES pressure and the normal frequency of transient LES relaxation^[22]. Long-term PPI therapy can aggravate atrophic gastritis in patients infected with *H.*

pylori. For *H pylori*-positive patients with gastroesophageal reflux disease, long-term PPI therapy should be preceded by the eradication of *H pylori*. During the long-term PPI therapy for GERD, *H pylori* infection can speed up the progress of gastric atrophy. Some investigators have proposed that *H pylori* should be eradicated in these patients. Nevertheless, eradication of *H pylori* does not have a clear effect on reflux symptoms in some GERD patients. A study by Spanish scientists showed that treatment of non-erosive gastroesophageal reflux disease with lansoprazole has no effect on *H pylori* infection^[23]. According to the randomized controlled trial by Schwizer *et al*^[24], symptoms of *H pylori*-positive GERD patients occur earlier than *H pylori*-negative patients and those on eradication therapy for *H pylori* infection, suggesting that *H pylori* increases the sensitivity of esophagus and accelerates recurrence of symptoms. In contrast, Moayyedi *et al*^[25] have not found any significant differences in the recurrence after eradication of *H pylori* in a large sample of patients. It was also reported that the infection rate of *H pylori* is higher in Chinese than in white Americans^[26,27].

Esophageal hiatal hernia is diagnosed mainly based on the upper gastrointestinal contrast. Although no recognized standards are available for endoscopic diagnosis of EHH, we can find some specific changes in EHH at endoscopic examination, including upward shift of the dentate line, hernia sac under intra-gastric reversal endoscope and gastric mucosa appearance within hernia sac. In the present study, the detection rate of esophageal hiatal hernia in those with reflux esophagitis was 13.9%. Esophageal hiatal hernia in patients with reflux esophagitis was not associated with *H pylori* infection or gender. The age of patients with reflux esophagitis and esophageal hiatal hernia was higher than that of those with simply reflux esophagitis. Esophagitis in patients with associated esophageal hiatal hernia was more serious. It was reported that hiatal hernia (HH) is closely associated with GERD, and isolated distal esophageal reflux is seen more in patients with HH than in patients without HH^[28]. Hiatal hernia, in combination with other reflux conditions and symptoms, is associated with the risk of esophageal adenocarcinoma^[29]. It was reported that no single factor or combined factors are capable of predicting mucosal damage with clinically sufficient certainty^[30].

In the present study, most patients with grade A of RE had one mucosal damage while those with grade B of RE had 2 or more mucosal damages. It was found that the most frequent location of mucosal damages in reflux esophagitis patients was the right esophageal wall, especially at the points of 2:00 and 4:00. This pathological change may be due to the anti-reflux role of oesophagogastric angle (His angle) and Gubaroff valve, which makes the left esophageal wall suffer from less gastric acid erosion. In contrast, the right esophageal wall is eroded and damaged by gastric contents more easily because of its direct connection with cardia ventriculi and the lack of valvar protection. Katsube *et al*^[31] reported that the circumferential location of esophageal mucosal breaks differs significantly among different grades of esophagitis, suggesting that reflux of gastric contents into the esophagus can be effectively improved after a valve is added to cardia ventriculi by means of endoscopy or surgical technique.

In conclusion, the peak age of RE onset is 40-60 years and higher in male than in females. The mean age of RE onset is lower in males than in females. The infection rate of *H pylori* is significantly lower in patients with esophagitis, but the severity of esophagitis is not associated with *H pylori* infection. Old age and combined esophageal hiatal hernia are associated with more severe esophagitis. Right esophageal mucosal damage can occur more often in patients with reflux esophagitis.

COMMENTS

Background

Gastroesophageal reflux disease (GERD) affects at least 5%-7% of the global population. The characteristics of GERD of white and yellow race are different. The characteristics of GERD in white people have been described, but the characteristics of GERD in Chinese are not sufficiently described.

Research frontiers

The clinical and endoscopic features of Chinese patients with reflux esophagitis (RE) were analyzed. The relationship between RE and patient's gender and age, between RE and *H pylori* infection, between RE and hiatal hernia (HH) was discussed. The main location of esophageal mucosa damages to esophageal wall was found.

Innovations and breakthroughs

The clinical and endoscopic features of Chinese patients with reflux esophagitis (RE) were analyzed. The peak age of RE onset was 40-60 years and higher in males than in females. The mean age of RE onset was lower in males than in females. The infection rate of *H pylori* was significantly lower in patients with esophagitis, but the severity of esophagitis was not associated with *H pylori* infection. Old age and combined esophageal hiatal hernia were associated with more severe esophagitis. Right esophageal mucosal damage can occur more often in patients with reflux esophagitis.

Applications

The characteristics of Chinese patients with RE were compared to those of people in other countries. Based on the fact that "right esophageal mucosal damage can occur more often in patients with reflux esophagitis", new methods to cure GERD with endoscopy or surgery should be recommended.

Peer review

In this manuscript, the authors analyzed the clinical and endoscopic features of Chinese patients with reflux esophagitis (RE) and described the low infection rate of *H pylori* in RE patients, the association of hiatal hernia with the severity of RE, as well as the prevalence of right-sided esophageal mucosal damage. The study was well designed and the conclusion was reliable. It should be noted the distribution of mucosal damage locations in patients with RE was not similar.

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CLINICAL RESEARCH

Cost-effectiveness analysis of early veno-venous hemofiltration for severe acute pancreatitis in China

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hemofiltration as an alternative therapy for SAP remains controversial. However, we propose that early use of short-term high-volume veno-venous hemofiltration would have a beneficial impact on the management of SAP.

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Key words: Veno-venous hemofiltration; Severe acute pancreatitis; Early management; Cost-effectiveness; Health economics

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Abstract

AIM: To determine the most cost-effective hemofiltration modality for early management of severe acute pancreatitis (SAP) in China.

METHODS: We carried out a search of Pub-Medline and Chinese Biomedical Disk database. Controlled clinical trials on Chinese population were included in the analysis. The four decision branches that were analyzed were: continuous or long-term veno-venous hemofiltration (CVVH/LVVH), short-term veno-venous hemofiltration (SVVH), SVVH plus peritoneal dialysis (PD), and non-hemofiltration control group. The effectiveness of the technique was determined by survival rate, complications prevention and surgery preservation. The total cost of hospitalization was also assessed.

RESULTS: The SVVH only technique was the least costly modality, \$5809 (44449 RMB), and was selected as the baseline treatment modality. SVVH only arm achieved the lowest C/E ratio in terms of overall survival, complications prevention and surgery preservation. In incremental cost-effectiveness analysis, the CVVH/LVVH only and the control arms were inferior to other techniques. Sensitivity analysis showed SVVH only and SVVH plus PD arms overlapped in C/survival ratio.

CONCLUSION: The role of early veno-venous

INTRODUCTION

Severe acute pancreatitis (SAP) is a grave illness associated with serious pancreatic and systematic disease. SAP is seen in nearly 20% of all patients with acute pancreatitis^[1]. Despite advances in the understanding of the pathophysiology and management of acute pancreatitis over the past several decades, the mortality rate of SAP has not shown a substantial decrease, varying from 8%-15% to more than 30% in some studies^[1-4]. The main factors that influence the poor outcome include systematic inflammatory response syndrome (SIRS) in the early stages (≤ 14 d) and infection of pancreatic and peri-pancreatic necrotic tissue in the late stages (> 14 d), both of which can precipitate secondary multi-organ deficiency syndrome (MODS)^[5]. As some reports indicate, at least 50% of deaths in the early stage of SAP are related to MODS, and when three or more organs fail, the mortality rate increases to 95%^[5,6]. Thus, efficient management during the early stages of the illness is important in improving the prognosis.

Since 1991, veno-venous hemofiltration (VVH) has been used in the initial management of SAP^[7]. Several studies have indicated that hemofiltration removes from the circulation small and medium sized molecules that stimulate the inflammatory cells. Alternatively, VVH

may directly inhibit the cells that contribute to the systematic response^[8]. The use of continuous veno-venous hemofiltration (CVVH) has been assessed in a animal model of SAP and was found to significantly improve the survival time, when used both for therapeutic and prophylactic treatment, especially the latter^[9]. However, the efficiency of treatment decreased with continuing use of CVVH, suggesting that the filter membranes were compromised by long-term application^[9]. The current consensus in Japan is to start CVVH soon after the onset of SAP, and to use it continuously for 3-14 d, because reduction in the chemical mediators, and improvement in the respiratory function and the incidence of MODS were more obvious if the treatment was started early rather than at a late stage^[10]. However, there is no consensus on how long CVVH should be used and when it should be stopped. Therefore, early short-term veno-venous hemofiltration (SVVH) modalities have been examined, including the use of repeated SVVH (RSVVH), intermittent SVVH (ISVVH) and single SVVH (SSVVH). The time interval of hemofiltration plays an important role in the treatment of SAP during its early stage. A comparison of SVVH with prolonged time interval VVH, and long-term veno-venous hemofiltration (LVVH) in the treatment of SAP did not improve the prognosis further but was associated with more side-effects^[11]. Therefore, in the decision making process the benefits of CVVH/LVVH and SVVH continue to be controversial. In addition, it should be noted that peritoneal dialysis (PD) is another approach to the treatment of SAP as it removes dialyzable toxins and reduces severe metabolic disturbances^[12]. In China, PD has also been used as an additional therapy with early SVVH. Clinical studies in China have reported that the use of early SVVH plus PD results in better prognosis of SAP, since cytokines such as TNF, IL-6 and IL-8 can be removed effectively from the circulation by these techniques^[13].

In China, which is a developing country with a huge population and relatively low income, the use of early CVVH/LVVH carries a great economic burden because of its high cost. For this reason, early SVVH may be more acceptable. However, which one of these therapeutic modalities provides reasonable effectiveness at a lower cost needs to be further explored. The present cost-effectiveness analysis is based on a review of the literature, with a view to determine as to which approach is the most cost-effective treatment of SAP in China.

MATERIALS AND METHODS

The model

The cost-effectiveness analysis was based on a decision tree designed to simulate a simplified clinical course of SAP treated with or without early VVH (Figure 1). In the general structure of the tree, there were four intervention decision arms: conventional therapy without hemofiltration (control arm), conventional therapy combined with early CVVH or LVVH (CVVH/LVVH only), SVVH only, and SVVH plus PD. All the hemofiltration modalities were started in the early stage of SAP, generally 3-5 d after onset of the disease.

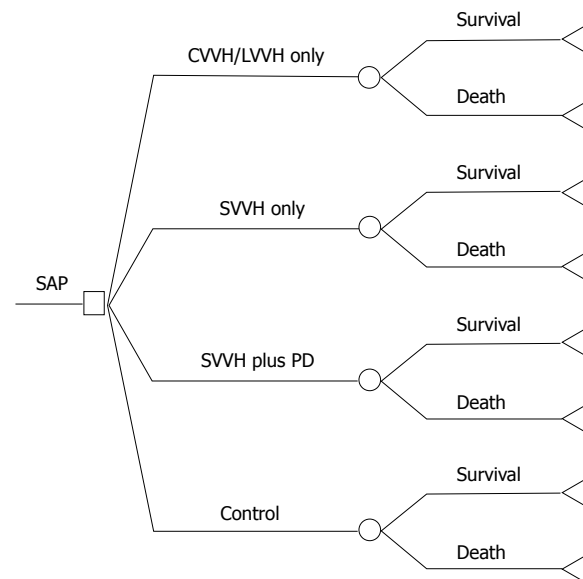


Figure 1 Decision tree of early hemofiltration for SAP.

Effectiveness data

The primary effectiveness variable was overall survival rate. The secondary effectiveness variables were the overall complication prevention rate and overall surgery preservation rate since complications and surgery were the factors most likely to increase the cost of hospitalization. The complications analyzed included severe local and systematic infections, and MODS. Surgery was mainly performed for necrosectomy. Thus, the primary clinical outcome measure was survival (alive = 1, death = 0), while the secondary outcome measures were complications (none = 1, occurrence = 0) and surgery (no = 1, yes = 0). The specified probabilities were retrieved from our previous systematic review (Table 1)^[14].

Cost data

The direct health care cost, i.e. total cost of hospitalization (currency, RMB), was calculated as mean \pm SD obtained from each of the studies, and the weighted costs were combined by the formula $\Sigma(\chi_i n_i) / \Sigma(n_i)$, of which χ = total cost of hospitalization, n = number of assigned patients in any of the intervention arms, and i = number of included studies. All the costs were converted to the price index as of 2005, taking into account the annual increase in the Chinese prices, i.e. 1.0% in 1999-2000, 2000-2001, 2001-2002 and 2002-2003, 1.2% in 2003-2004, and 3.9% in 2004-2005^[15]. One RMB was converted to 0.130688 U.S. dollars.

Literature search and selection

We searched Pub-Medline and Chinese Biomedical Disk (CBMdisc) database from 1990 to 2006. The search strategy was combining the subheading and text words hemofiltration and pancreatitis. The studies based on Chinese population were selected regardless of the language. All patients were diagnosed to have SAP based on the Atlanta classification, APACHE II score > 8 , Ranson score > 3 , or Balthazar CT grading of D or E. All clinical studies which assessed cost comparing

Table 1 Outcomes based on a previous systematic review^[14]

Outcomes	CVVH /LVVH only		SVVH only		SVVH plus PD		Control	
Overall mortality rate (% , ratio)	0.149	40/47	0.058	129/137	0.147	29/34	0.179	322/392
Survival rate (%)	0.851		0.942		0.853		0.821	
Overall complications rate (% , ratio)	0.267	4/15	0.208	15/72	0.157	8/51	0.412	120/291
Complications prevention rate (%)	0.733		0.792		0.843		0.588	
Surgery rate (% , ratio)	0.075	3/40	0.016	1/62	0.082	5/61	0.294	58/197
Surgery preservation rate (%)	0.925		0.984		0.918		0.706	

CVVH: Continuous veno-venous hemofiltration; LVVH: Long-term veno-venous hemofiltration; SVVH: Short-term veno-venous hemofiltration; PD: Peritoneal dialysis.

Table 2 Total hospitalization costs obtained from published Chinese articles (10 000 RMB)

Study	Refence	CVVH/LVVH only			SVVH only			SVVH plus PD			Control		
		Mean	SD	n	Mean	SD	n	Mean	SD	n	Mean	SD	n
Mao EQ 1999	[23]	-	-	-	5.32	1.6	10	-	-	-	8.91	2.5	10
Mao EQ 2003	[19]	13.7	10.5	16	5.66	5.64	20	-	-	-	-	-	-
Feng GH 2004	[21]	-	-	-	-	-	-	6.1	1.9	25	9.4	3.1	15
Yang FF 2005	[24]	-	-	-	-	-	-	5.8	2.2	36	10.2	4.3	64
Zhang T 2005	[25]	-	-	-	3.29	1.279	38	-	-	-	6.884	4.868	71

CVVH: Continuous veno-venous hemofiltration; LVVH: Long-term veno-venous hemofiltration; SVVH: Short-term veno-venous hemofiltration; PD: Peritoneal dialysis.

Table 3 Weighted total hospitalization costs

Modalities	Cost (C) ¹			Incremental cost (ΔC) ²
	WM	Max	Min	
CVVH/LVVH only	\$18 826 (144 051 RMB)	\$33 254 (254 455 RMB)	\$4397 (33 647 RMB)	\$13 017 (99 602 RMB)
SVVH only	\$5809 (44 449 RMB)	\$9359 (71 613 RMB)	\$2259 (17 286 RMB)	
SVVH plus PD	\$7868 (60 205 RMB)	\$10 622 (81 279 RMB)	\$5114 (39 130 RMB)	\$2059 (15 756 RMB)
Control	\$11 317 (86 597 RMB)	\$17 006 (130 128 RMB)	\$5628 (43 066 RMB)	\$5508 (42 148 RMB)

¹All the costs were converted to 2005 price before combination. ²SVVH only, the least costly arm, was selected as common baseline for other arms to reference to. WM: Weighted mean; WMD: Weighted mean difference; CVVH: Continuous veno-venous hemofiltration; LVVH: Long-term veno-venous hemofiltration; SVVH: Short-term veno-venous hemofiltration; PD: Peritoneal dialysis.

hemofiltration with either a control group or another treatment modality were eligible for inclusion in the analysis.

Statistical analysis

TreeAge Pro Healthcare 2006 software was used in modeling and analyses. Both the cost-effectiveness analyses and the incremental cost-effectiveness analyses were examined, with C/E ratio and incremental C/E (ΔC/ΔE) ratio calculated separately. The treatment arm with the lowest cost was selected as the common baseline for comparison with other treatment arms. If there was uncertainty with regard to decision making, sensitivity analysis was carried out by alternating the variables to maximal and minimal limits (two-way), including the total cost of hospitalization and overall survival rate.

RESULTS

Our previous systematic review analyzed 10 randomized controlled trials and 6 clinical controlled trials comprising of a total of 891 Chinese patients^[14]. The meta-analysis

showed that the overall mortality rate was significantly reduced in the hemofiltration group [RR = 0.49, 95% CI (0.32, 0.74), $P = 0.0008$]^[14]. The specified probabilities of the clinical outcomes of each treatment arm were retrieved based on the sub-group analysis of the systematic review. The overall survival rates improved in the CVVH/LVVH only, SVVH only and SVVH plus PD arms by diverse extent (Table 1). Five controlled studies from 4 Chinese medical institutions which provided the data on cost were included in the present analysis^[11,13,16-18], and the detailed data were extracted and combined (Tables 2 and 3). Interestingly, both SVVH only and SVVH plus PD reduced the total cost of hospitalization compared with the control arm, while the CVVH/LVVH only approach was the most costly. By contrast, the SVVH only approach was the least costly arm, and was therefore selected as the baseline for the purpose of comparing the cost-effectiveness and incremental cost-effectiveness with the other treatment arms.

Cost-effectiveness analysis

In the cost-effectiveness analysis, the lowest ratios C/

survival rate, C/complication prevention rate and C/surgery preservation rate were \$6167 (47186 RMB), \$7334 (56122 RMB) and \$5903 (45172 RMB) respectively in SVVH only arm. These findings indicate that a patient treated with SVVH would pay an additional \$62 (472 RMB), \$73 (561 RMB) and \$59 (452 RMB) respectively to gain 1% higher probability of each benefit. To summarize, the cost-effectiveness analysis can be ranked in the order of superior to inferior as SVVH only, SVVH plus PD, control and CVVH/LVVH only (Table 4).

In incremental cost-effectiveness analysis, the CVVH/LVVH only, SVVH plus PD and control arms were inferior to SVVH only arm in outcomes of overall survival and overall surgery preservation, while the CVVH/LVVH only and control arms were inferior to SVVH only and SVVH plus PD arms in the aspect of overall complication prevention (Table 4, Figure 2). The incremental C/complication prevention ratio of SVVH plus PD arm was \$40385 (308941 RMB), which suggest that a patient treated with SVVH plus PD will pay an additional \$404 (3089 RMB) compared to SVVH only to obtain a 1% higher probability of preventing complications.

Sensitivity analysis

SVVH plus PD was the closest to SVVH only in both cost and effectiveness. Therefore, we performed sensitivity analysis to compare SVVH only and SVVH plus PD arms by changing the survival rate and cost in their ranges (Figure 3). The variable range of survival rates of SVVH only and SVVH plus PD arms were 0.900-1.000 and 0.853-1.000 respectively, which were retrieved from our previous meta-analysis^[14]. The range of cost is listed in Table 3. It is clear that there is overlapping in the variable areas of the two modalities. The minimal and maximal C/survival rate ratios were \$2259 (17286 RMB) - \$10399 (79570 RMB) and \$5114 (39130 RMB) - \$12453 (95286 RMB) in SVVH only and SVVH plus PD arms respectively. If in area A, SVVH only was superior to SVVH plus PD modality, the ratio of C/survival rate was less than \$5114 (39130 RMB). By contrast, if in area B, SVVH plus PD was inferior to SVVH only, the ratio of C/survival rate was more than \$10399 (79570 RMB).

DISCUSSION

Persistent SIRS, which is an early sign of SAP, is associated with MODS and even death^[19]. About 50% of deaths in patients with SAP occur in the early stage of the disease; these patients experience a severe initial attack and develop an exaggerated SIRS with the development of MODS and death^[20]. Therefore, several treatment modalities which target the inflammatory response in patients with SAP have been under consideration^[21].

The onset and subsequent rapid deterioration seen in SAP is likely due to the over-production of pro-inflammatory cytokines, which are considered critical to the pathogenesis of the disease^[14]. Thus, cytokines derived from macrophages are believed to play an integral role in the evolution of acute pancreatitis^[22]. It has been suggested that the local pancreatic lesion activates macrophages to release pro-inflammatory cytokines^[14,23]. This results in an

Table 4 Results of cost-effectiveness analyses (\$/%)

Subsets	CVVH/LVVH only	SVVH only	SVVH plus PD	Control
C/Survival	\$22122	\$6167	\$9224	\$13785
C/Complication prevention	\$25683	\$7334	\$9333	\$19247
C/Surgery preservation	\$20352	\$5903	\$8571	\$16030
ΔC/ΔSurvival	Dominated	-	Dominated	Dominated
ΔC/ΔComplication prevention	Dominated	-	\$40375	Dominated
ΔC/ΔSurgery preservation	Dominated	-	Dominated	Dominated

CVVH: Continuous veno-venous hemofiltration; LVVH: Long-term veno-venous hemofiltration; SVVH: Short-term veno-venous hemofiltration. SVVH only, the least costly arm, was selected as common baseline for other arms to reference to; PD: Peritoneal dialysis.

imbalance between pro- and anti-inflammatory cytokines, resulting in the development of SIRS. Some mediators, such as TNF- α , phospholipase, and kinin, are increased greatly in animal models of SAP^[9], and some studies have shown that there is a significant correlation between the serum levels of IL-1- β , IL-6, IL-8, IL-10 and IL-11 and the severity of acute pancreatitis^[24-27]. Animal studies have shown that early blockade of the cytokine cascade at the level of the IL-1 receptor significantly decreases the severity of pancreatitis and intrinsic pancreatic damage, as well as the mortality from SAP^[28,29]. Several antagonists of the inflammatory mediators have been used successfully in the laboratory setting and are currently being examined in prospective randomized trials^[30]. The effectiveness of any antagonist depends not only on its ability to block the effects of the inflammatory mediators but also on its administration early enough in the course of the disease, before pancreatic necrosis and organ dysfunction sets in^[30]. Thus, the inhibition of the cytokine cascade should potentially alleviate the pancreatic and systematic inflammation and improve the outcome of SAP.

The present cost analysis was carried out to determine the most economical and effective hemofiltration modality in China. CVVH has been considered as a potentially effective approach in the management SAP for nearly a decade. However, in China, both CVVH and LVVH are too costly for the common public. Thus, the less costly approach, SVVH was analyzed. Several clinical studies of SVVH administrated to patients with SAP in the early stage of the disease have been carried out in various institutions in China. However, these studies had limited scale of participation, were methodologically of poor quality (only a few studies were randomized) and very few discussed the cost outcomes. Therefore, our results may be biased by these confounding factors.

Our previous meta-analysis, based on controlled trials carried out in China indicated that early SVVH was effective and safe for SAP, but the efficacy of CVVH/LVVH could not be proven^[14]. Our initial findings inspired us to explore further the role of other treatment modalities in decision making. The results of the present analysis showed that SVVH is the most suitable approach

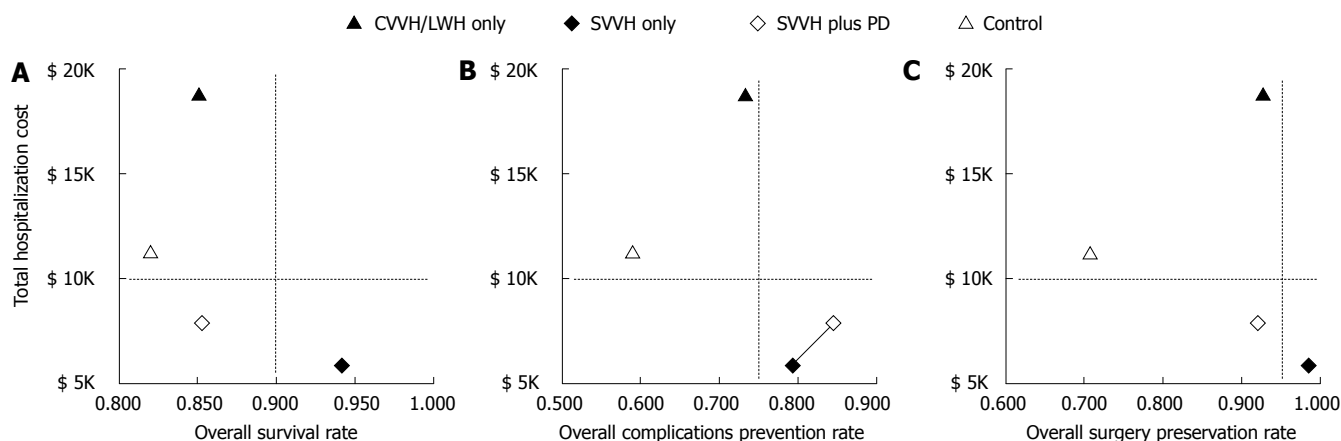


Figure 2 Cost-effectiveness analysis plots. **A:** Cost-survival ratio; **B:** Cost-complications prevention ratio; **C:** Cost-surgery preservation ratio.

in the treatment of SAP in China. The use of SVVH only would result in the best clinical outcome with reduction in the overall mortality, and prevention of complications and surgery. Furthermore, SVVH only modality is the least costly compared to other treatment options, including CVVH/LVH only, SVVH plus PD and non-hemofiltration modalities. The CVVH/LVH only and non-hemofiltration modalities were surpassed by SVVH only. The SVVH plus PD modality was fairly close to SVVH only in efficacy and cost, but SVVH was superior. However, the sensitivity analysis showed overlapping of the cost-effectiveness ratio between SVVH only and SVVH plus PD modalities. These findings suggest that SVVH is not entirely superior to SVVH plus PD, and SVVH plus PD should be considered as a suitable alternative option in China, and requires further investigation about its cost-effectiveness.

The timing of hemofiltration is considered as a critical factor in the outcome of patients with SIRS or sepsis. The subset of patients with these complications may benefit from the use of early short-term pulse hemofiltration^[31]. The use of LVVH did not improve the prognosis but was associated with more side-effects than SVVH^[11]. Another factor which may influence the outcome of patients with SIRS or sepsis is the ultrafiltration rate. It has been noted that the beneficial effects are greater with “very high” ultrafiltration rates (≥ 100 mL/kg per hour)^[31].

The benefit of VVH remains to be defined as an alternative therapy for SAP with SIRS or sepsis. A definite conclusion cannot be drawn because the studies have been small, are of poor quality and are heterogeneous in nature. Thus, there is no evidence in humans to recommend the use of VVH as an adjuvant therapy in patients with SAP. There continues to be uncertainty about the absolute indication, timing interval, dosing volume and the type of membrane required. Therefore, more randomized clinical trials are required before definite recommendations can be made about the clinical management of SAP. However, based on the present cost-effectiveness analysis in China, we suggest that the use of early short-term high-volume VVH is likely to play an important role in the management severe acute pancreatitis accompanied with SIRS, sepsis or organ failure.

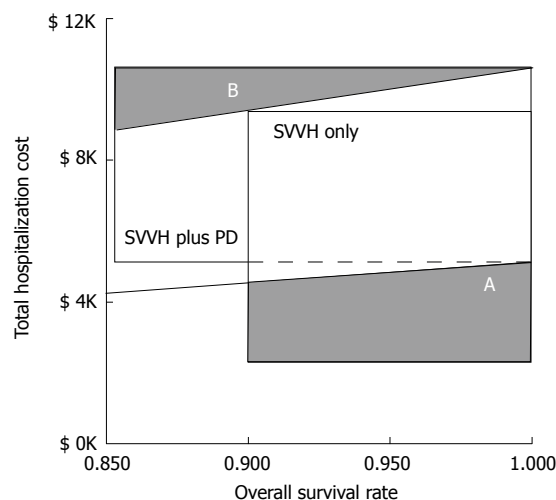


Figure 3 Two way sensitivity analysis of C/E ratio between SVVH only and SVVH plus PD modalities.

COMMENTS

Background

Nearly 50% of deaths in severe acute pancreatitis (SAP) occur during the early stage of the disease. These patients experience a severe initial attack and develop an exaggerated systemic inflammatory response syndrome (SIRS), with the development of multiple organ dysfunction syndrome (MODS) and death. Therefore, the role for therapy targeting the inflammatory response in SAP has been under much consideration recently.

Research frontiers

The onset and the poor outcome of SAP is likely due to the over-production of pro-inflammatory cytokines, which is considered as the critical factor in this condition. Inhibition of the cytokine cascade should potentially alleviate the pancreatic and systematic inflammatory response, and improve the outcome of SAP. Thus, veno-venous hemofiltration which can effectively eliminate the cytokines, has been used in the early management of SAP. Several different modalities of hemofiltration are available, but their effectiveness is controversial. In particular, expensive treatments should be used with much discretion in China, a developing country.

Innovations and breakthroughs

Based on our previous meta-analysis, early veno-venous hemofiltration may significantly reduce the overall mortality compared to no treatment. Analysis of different modalities showed that continuous or long-term veno-venous hemofiltration (CVVH/LVVH) and short-term veno-venous hemofiltration plus peritoneal dialysis (SVVH + PD) do not reduce the mortality significantly, whereas short-term only modality (SVVH only) was superior to other treatments in this

respect. A cost-effectiveness analysis based on the Chinese literature showed that SVVH only was the most cost-effective modality in reducing mortality, and in preventing complications and surgery. It can be implied that the timing of veno-venous hemofiltration should be regarded as a critical factor in the outcome of patients with SIRS or sepsis.

Applications

Early veno-venous hemofiltration is considered as an effective alternative therapy for SAP, although it is expensive for the general population in China. Based on the current evidence, hemofiltration can control to a certain extent SIRS and even MODS, if used during the early stage of the disease, with SVVH only the most cost-effective modality in China. We believe that early short-term high-volume veno-venous hemofiltration will play an important role in the management of SAP with SIRS, sepsis or organ failure.

Terminology

Severe acute pancreatitis (SAP) is a serious disease with intense pancreatic and systematic inflammation, seen in about 20% of patients with acute pancreatitis. Veno-venous hemofiltration removes waste products including cytokines by passing the blood through extracorporeal filters in the veno-venous access, which is categorized as continuous, long-term and short-term modalities based on the duration of hemofiltration.

Peer review

It is a well written article dealing with the cost-effective of veno-venous hemofiltration in the early treatment of SAP.

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RAPID COMMUNICATION

Factors influencing a low rate of hepatitis C viral RNA clearance in heroin users from Southern China

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Abstract

AIM: To study the virological and host factors influencing hepatitis C infection outcomes in heroin users in southern China.

METHODS: HCV RNA and associated factors were analyzed among 347 heroin users from Guangxi Zhuang Autonomous Region, southern China who were hepatitis C virus (HCV) EIA positive for two or more consecutive visits.

RESULTS: Using the COBAS AMPLICOR HCV TEST, a remarkably low HCV RNA negative rate of 8.6% was detected. After multivariate logistic regression analysis, HCV RNA clearance was significantly associated with the presence of HBsAg (OR = 8.436, $P < 0.0001$), the lack of HIV-1 infection (OR = 0.256, $P = 0.038$) and age younger than 25 (OR = 0.400, $P = 0.029$).

CONCLUSION: Our study suggests HCV infection among Chinese heroin users results in high levels of viral persistence even amidst factors previously found to enhance viral clearance. Prospective studies of a possible genetic component within the Chinese population and the pathogenicity of non-genotype 1 HCV infections are needed.

INTRODUCTION

The hepatitis C virus (HCV) epidemic now affects over 200 million people worldwide. HCV is a single-stranded RNA flavivirus that is responsible for the majority of non-A-non-B hepatitis infections^[1,2]. Natural history studies have found that 15%-59% of people who are infected with HCV will undergo spontaneous viral clearance with no further liver disease due to HCV^[3-11]. The remaining will develop chronic HCV infection that can lead to cirrhosis, hepatocellular carcinoma and the need for a liver transplant.

The exact mechanism of HCV RNA clearance is not well understood although recent studies have shown that clearance is associated with strong, broad cellular immune responses^[12-19]. Other factors such as younger age^[3,20], female gender^[21,22], presence of hepatitis B surface antigen (HBsAg)^[3,23], certain HLA alleles^[6,24-29], and low viral quasispecies diversity^[30] have been linked to increased HCV RNA clearance. While African American ethnicity^[3,25], HCV genotype 1^[31-33] and co-infections with human immunodeficiency virus-1 (HIV-1)^[3], Human T-Lymphotropic Virus-1^[34] and *Schistosoma mansoni*^[35] have been associated with lower HCV RNA clearance and higher HCV RNA levels. Understanding why and how individuals clear HCV is the key to developing new drugs and an effective vaccine^[21].

Studies of heroin users from Guangxi Zhuang Autonomous Region in southern China have attributed the appearance and spread of HIV, HCV and other infectious agents to the change in heroin using patterns from smoking to injection^[36]. In Guangxi, two separate emerging HIV epidemics have sprung from different

heroin trafficking routes into the province^[37,38]. It is not clear how widespread the HCV epidemic is in China. Little seroprevalence data exists, mostly from the major cities of Beijing, Shanghai and Hong Kong and to our knowledge; no studies on HCV RNA clearance in China have been done. Studies in Yunnan Province, which borders Guangxi Zhuang Autonomous Region in the west, have found high HCV co-infection rates in HIV-1 positive drug users^[39]. Previous epidemiological studies in Guangxi not only found similar high rates of HCV co-infection in HIV-1 positive drug users, but a high HCV prevalence (72%), incidence (37.8 per 100 person years) in all heroin users enrolled in the study^[40]. To determine the clearance rate of HCV RNA and study factors that influence clearance, serum from a large cohort of heroin users from Guangxi Zhuang Autonomous Region, southern China was qualitatively tested for HCV RNA.

MATERIALS AND METHODS

Study participants

Heroin users over the age of 18 in Guangxi Zhuang Autonomous Region, China are currently being followed in a study of behavioral and virological features of HIV-1 in injection drug users conducted at the Guangxi Provincial Health and Anti-Epidemic Center. Over 600 participants are being followed at study sites in Pingxiang and Binyang City in the Guangxi Zhuang Autonomous Region. Study participants from Pingxiang have been followed since September 1999 while participants from Binyang have been followed since January 2000. The informed consent procedure was described previously^[40]. Briefly, at each visit, participants underwent blood draw and personal interviews and were counseled on the results of their serological tests. Baseline and follow-up questionnaires entailed a brief medical history including sexually transmitted diseases, history of drug use, sexual history, ethnic and economical backgrounds. Blood was collected and centrifuged and the serum either underwent serological assays or was stored at -70°C. Samples were then shipped from Guangxi to our laboratory in Baltimore, MD for HCV RNA testing and further analysis.

Serologic assays

At the Guangxi Health and Anti-Epidemic Center in Nanning, the presence of HIV-1 antibody was determined by enzyme-linked immunosorbent assay (ELISA) using the Vironostika HIV-1 Microelisa System (Organon Teknika). All ELISA-positive samples were not considered HIV-positive until confirmation by HIV-1/2 Western blot immune assay manufactured by Gene Lab (Singapore). Positivity for Hepatitis B surface antigen (HBsAg) and antibody to Hepatitis B surface antigen (HBsAb) were determined by HBV ELISA (Xiamen Xinchung Scientific, Xiamen, China). Hepatitis C antibody was analyzed by the Ortho HCV Version 3.0 ELISA Test System (Ortho Diagnostic Systems, Raritan, NJ).

HCV RNA detection and determination of HCV serotypes and genotypes

Available sera from 347 participants who were HCV

antibody positive for two consecutive visits (> 6 mo apart) were qualitatively tested for Hepatitis C RNA by the COBAS AMPLICOR HCV TEST KIT, sensitivity > 50 IU/mL (Version 2.0, Roche Diagnostics). Hepatitis C serotypes were determined for samples found HCV RNA negative using the Murex HC03 ELISA (Abbott Diagnostics, England). RNA for HCV genotyping was extracted from 100 µL of serum using the QIAamp Viral RNA kit (QIAGEN Inc, Valencia, CA). Reverse transcription and nested PCR was performed using primers to conserved regions of Core and E1 as previously described^[41]. After purification with the QIAquick PCR Purification kit (QIAGEN Inc, Valencia, CA), samples were sequenced using the inner forward primer on an automated sequencer (PRISM, version 2.1.1; ABI, Foster City, CA). Sequences were compiled using the BioEdit program, version 4.7 (T. Hall, North Carolina State University, Raleigh) and genotypes were assigned after alignment with known HCV genotypes as previously described^[41].

Statistical analysis

Univariate logistic regression analyses were first performed to explore the crude associations between the clearance of HCV and related factors, including age, length of drug use, frequency of drug use, injection drug use, HIV-1 Ab, HBsAb, HBsAg, study site, ethnicity and gender. Variables with a $P < 0.1$ in the univariate model were then put into a multiple logistic regression model^[42]. Those that ceased to be significant in the multivariate model, $P > 0.05$, were eliminated in a stage-wise manner, yielding a final model in which all variables were independently associated with the clearance of HCV. P -values reported are two-sided. The age, frequency of drug use and length of drug use closest to the overall sample mean within a factor of five were used in the model.

RESULTS

A total of 347 study participants who were HCV EIA positive for two or more consecutive study visits were included in this study, 127 from Pingxiang City and 220 from Binyang City. Pingxiang City is in southern Guangxi and borders Vietnam while Binyang City is centrally located within the province. Survey and serology results from the study visit of HCV RNA analysis are listed in Table 1. This subset of the Guangxi cohort is predominantly male (96.25%) with a mean age of 27 (range 19-50). Two main ethnic groups are present in Guangxi, Han and the Zhuang minority. Approximately 67% of the study group is Han and 29% Zhuang. Less than half of the participants are married (32%). Over 90% of the participants have a middle school or lower level of education.

Approximately 93% of the heroin users admit to injection drug use (Table 1). Over half admit to sharing needles (data not shown). The participants have been injection drug users for an average of about 5 years (Table 1). The study group uses heroin at an average frequency of 74 times per month. Along with being HCV EIA positive, 25.94% of the study participants are also HIV Ab positive,

Table 1 Characteristics of consecutively HCV ELISA positive heroin users from Guangxi Zhuang Autonomous Region, China

	Number (%) HCV RNA		Total
	(+)	(-)	
Factor	317 (91.35)	30 (8.65)	347
Location			
Binyang	200	20	220
Pingxiang	117	10	127
Mean age (yr)			
Mean \pm SD	27.5 \pm 5.7	25.4 \pm 4.52	27.4 \pm 5.6
Range	(19-50)	(19-37)	(19-50)
Gender			
Male	307 (96.85)	27 (90.00)	334 (96.25)
Female	10 (3.15)	3 (10.00)	13 (3.75)
Ethnicity			
Han	212 (66.88)	20 (66.67)	232 (66.86)
Zhuang	94 (29.65)	7 (23.3)	101 (29.11)
Other	11 (3.47)	3 (10.0)	14 (4.03)
Marital status			
Single	215 (67.82)	21 (70.00)	236 (68.01)
Married	102 (32.18)	9 (30.00)	111 (31.99)
HIV Ab status			
Positive	87 (27.44)	3 (10.00)	90 (25.94)
HBsAg status			
Positive	32 (10.09)	14 (46.67)	46 (13.26)
HBsAb status			
Positive	142 (44.79)	6 (20.00)	148 (42.65)
Injection drug use			
Yes	294 (92.74)	29 (96.67)	323 (93.08)
Mean length of drug use			
Months \pm SD	63.7 \pm 26.4	54.72 \pm 28.2	62.9 \pm 26.6
Range	(9.0-165.2)	(13.1-124.2)	(9.0-165.2)
Mean frequency of drug use			
Per Month \pm SD	73.9 \pm 44.7	79.6 \pm 34.6	74.4 \pm 44.0
Range	(1.0-390.0)	(20.0-150.0)	(1.0-390.0)
Education level			
College or above	1 (0.32)	0	1 (0.29)
High school	26 (8.25)	2 (6.9)	28 (8.14)
Middle school	151 (47.94)	12 (41.38)	163 (47.38)
Primary school	133 (42.22)	15 (51.72)	148 (43.02)
Illiterate	4 (1.27)	0	4 (1.16)
Unknown	2	1	3 (0.86)

SD: Standard deviation; HCV: Hepatitis C virus; HIVAb: HIV-1 antibody; HBsAg: Hepatitis B surface antigen; HBsAb: Antibody to hepatitis B surface antigen.

13.26% are HBV surface antigen positive (HBsAg) and 42.65% are antibody positive for the HBV surface antigen (HBsAb). Of the 347 consecutively HCV EIA positive samples tested, only 30 had undetectable levels of HCV RNA (less than 50 IU/mL) resulting in an HCV RNA clearance rate of 8.6% (Table 1).

Results of univariate logistic regression analyses and final multivariate logistic regression analyses for Hepatitis C Viral RNA clearance are shown in Table 2. Univariate analysis revealed HCV RNA clearance to be associated with age younger than 25 (OR = 0.472), lack of HIV-1 infection (OR = 0.294), presence of HBsAg (OR = 7.793), lack of HBsAb (OR = 0.308), female gender (OR = 3.411) and acknowledgement of injection drug use (OR = 0.441). In the final model, only three factors were independently associated with HCV RNA clearance; being HBsAg

positive (OR = 8.436, $P < 0.0001$), lacking HIV-1 infection (OR = 0.256, $P = 0.038$) and age younger than 25 (OR = 0.400, $P = 0.029$).

A comparison of previously published HCV RNA clearance rate can be found in Table 3. Our HCV RNA clearance rate is most similar to African American injection drug users in the Baltimore ALIVE cohort^[3], but remarkably less than the remaining studies listed.

DISCUSSION

Injection drug practices in Guangxi Zhuang Autonomous Region, China continue to efficiently spread HCV, HIV and HBV resulting in large numbers of co-infections and multi-infections, the full impact of which has yet to be determined. Analysis of this cohort found a very low spontaneous HCV clearance rate (8.6%) among heroin users who have been HCV EIA positive for two or more consecutive study visits. The natural history of HCV in Chinese individuals has not been studied to this extent before.

Hepatitis C viral clearance was strongly associated with co-infection by the hepatitis B virus, specifically participants currently HBsAg positive. Other cohorts have seen trends between HBsAg positivity and HCV clearance^[3,23], but none with an association as convincing as is shown in our cohort. Previous studies of HBV and HCV co-infections have revealed viral interference between these hepatotropic viruses resulting either in one dominant virus^[43] or in some cases resolution of both infections^[44]. The exact mechanism of this interference is not known, although data suggest it is the result of inhibition of replication by viral proteins^[45,46]. It is also plausible that the existing activation of non-specific immune responses within the liver during the current HBV infection enhances the clearance of the HCV infection.

HCV is now a major opportunistic infection for those with co-infected HIV-1^[47]. Previous studies have shown that co-infections with HIV and HCV do not increase the progression to AIDS^[48], but do increase the HCV viral load and progression to end-stage liver disease (ESLD)^[49,50]. Two separate subtypes of HIV, A/E and a B/C recombinant, were likely to enter Guangxi Zhuang Autonomous Region in 1996^[38]. With high HCV prevalence and incidence rates, almost all injection drug users in Guangxi who become HIV-1 positive will be co-infected with HCV. This study found a significant association between HIV co-infection, defined by the presence of HIV-1 antibody, and the inability of the individual to clear HCV RNA. Only 3/90 HIV-1 antibody positive individuals were able to clear their HCV RNA. Two of these participants were new HIV-1 seroconverters and the third was HBsAg positive (data not shown). The mechanism of how HIV-1 infection inhibits HCV RNA clearance is likely due to immune suppression caused by HIV-1. It is less likely that the two HIV-1 seroconverters who cleared HCV RNA were immunocompromised (data not shown). Thomas *et al* did not see a significant association between HIV-1 co-infection and HCV clearance in the ALIVE cohort until HIV-1 infected people were broken down by CD4 levels^[3].

Table 2 Factors associated with hepatitis C viral clearance among 347 consecutively HCV antibody positive heroin users from Guangxi Zhuang Autonomous Region, China

Factor	Number (% clearance)	Univariate	Final multivariate model	
		Adjusted OR (95% CI)	Adjusted OR (95% CI)	P
Age (yr)				
≤ 25	125 (13.6)	1.0	1.0	0.029
> 25	222 (5.9)	0.472 (0.221-1.006)	0.400 (0.176-0.909)	
HIV Ab status				
Negative	257 (10.5)	1.0	1.0	0.038
Positive	90 (3.3)	0.294 (0.087-0.993)	0.256 (0.071-0.924)	
HBsAg status				
Negative	301 (5.3)	1.0	1.0	< 0.0001
Positive	46 (30.4)	7.793 (3.484-17.430)	8.436 (3.646-19.520)	
HBsAb status				
Negative	199 (12.1)	1.0		
Positive	148 (4.1)	0.308 (0.123-0.744)		
Gender				
Male	334 (8.1)	1.0		
Female	13 (23.1)	3.411 (0.885-13.143)		
Location				
Binyang	220 (9.1)	1.0		
Pingxiang	127 (7.9)	0.855 (0.387-1.888)		
Ethnicity				
Zhuang minority	115 (8.7)	1.0		
Han	232 (8.6)	0.991 (0.448-2.192)		
Injection drug use				
Yes	323 (9.0)	1.0		
No	24 (4.2)	0.441 (0.057-3.384)		
Length of drug use				
< 5 yr	176 (10.8)	1.0		
≥ 5 yr	171 (6.4)	0.568 (0.262-1.233)		
Frequency of drug use				
< 75 times per month	217 (7.4)	1.0		
≥ 75 times per month	130 (10.8)	1.516 (0.714-3.219)		

OR: Odds ratio; CI: Confidence Interval; HCV: Hepatitis C virus; HIVAb: HIV-1 antibody; HBsAg: Hepatitis B surface antigen; HBsAb: Antibody to hepatitis B surface antigen.

Age of the individual also affected the HCV clearance rate in our cohort. A meta-analysis by Mathei *et al* found a linear relationship between mean age and HCV RNA clearance rates^[20]. The higher prevalence of HCV RNA in older individuals was suggested as a result of continuous re-exposure to HCV for a prolonged period of time. The ages found in our cohort are much younger than previous HCV studies where HCV RNA clearance rates were less than 15%^[3,20]. We attempted to address whether our high levels of HCV RNA persistence were due to the length and frequency of drug use, but these factors proved non-significant.

HCV genotype 1 has been considered a more aggressive genotype, associated with lower clearance rates, decreased susceptibility to current treatments^[32-34] and in a few cases, associated with a faster progression of HIV disease^[51]. Many HCV cohort studies, including the ALIVE cohort in Baltimore are primarily genotype 1 infections^[5]. Examination of our cohort found three major HCV subtypes present in chronic infections, genotypes 6a (38%), 3b (37%) and 1a (19%)^[52]. It is unclear whether HCV genotypes in our cohort are responsible for the low HCV RNA clearance rate.

Age, HBsAg positivity and lack of HIV-1 co-infection were the most significant factors resulting in HCV RNA

clearance. In comparison with the ALIVE cohort^[3], our lower age, lower prevalence of HIV-1 and higher prevalence of HBsAg, would predict higher rates of HCV RNA clearance than was seen. After removing HIV-1 and HBsAg positive individuals, only 15 of the remaining 221 participants underwent HCV clearance, at a rate of 6.8% (data not shown). This suggests that other host and viral factors are present in the Guangxi cohort resulting in high rates of HCV persistence. Studies of the ALIVE cohort by Thomas *et al* found the lower HCV RNA clearance levels in African American injection drug users to be linked to differences in certain HLA-frequencies^[3,25]. There is also speculation of differences in TH1/TH2 cytokine balances between Caucasian and African Americans^[53]. Whether HLA or cytokine profiles of Chinese individuals account for the low HCV RNA clearance rates has yet to be seen.

Injection heroin use in China is rapidly distributing HCV, HBV and HIV. The natural history of HCV in Chinese heroin users results in little spontaneous clearance of HCV RNA. Current HIV infection further debilitates the individual's ability to control HCV infection. And although HBV is endemic in China and viral interference between HBV and HCV may eliminate one of the hepatropic viruses, it may not decrease the possibility for further liver disease. Further studies within the Guangxi

Table 3 HCV RNA clearance rates among previously published cohort studies

Principal investigator	Cohort	HCV RNA			
		Number tested	clearance rate (%)	Percent HIV Ab +	Percent HBsAg +
This study Thomas ^[3]	Chinese IDU	347	8.7 ¹	25.9%	13.3%
	African				
	American IDU	729	9.3		
	Non-African				
	American IDU	44	36.0		
Alric ^[5]	Overall	773	10.9 ¹	45.7%	3.5%
	Caucasian				
	French	123	25 ¹	ND	ND
Minton ^[6]	Caucasian				
	English	172	20.3 ¹	ND	ND
Yee ^[7]	English				
Alter ^[8]	Hemophiliacs	200	14 ¹	40.3%	ND
	American blood				
	Donors	248	14 ²	ND	ND
Kenny-Walsh ^[9] Alter ^[10]	(42% IDU)				
	Irish women	704	44.6 ²	ND	ND
	Non-Hispanic				
	African				
	American	196	14.0		
Sagnelli ^[11]	Non-Hispanic				
	Caucasian	119	32.0		
	Hispanic				
	Americans	132	26.0		
	NHANES				
	Overall	447	26.1 ²	ND	ND
	Italian liver				
	Patients	336	21.7 ³	ND	28%

¹HCV RNA by Roche AMPLICOR HCV Qualitative Assay-Sensitivity > 50 IU/mL; ²HCV RNA by In-house RT-PCR- Sensitivity Unknown; ³HCV RNA by HEPA-Check C - Sensitivity Unknown; HCV: Hepatitis C virus; IDU: Injection drug user; HIV: Human immunodeficiency virus 1; HBsAg: HBV surface antigen; ND: Not determined.

cohort and other Chinese Provinces will better define the pathogenesis of HCV in Chinese ethnicities and non-genotype 1 infections. In order to decrease the spread of HIV-1 and HCV, education on safe-needle practices and the illnesses transmitted by injection drug use is urgent in China.

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COMMENTS

Background

Hepatitis C virus (HCV) is quickly spread through injection drug use. A proportion of individuals infected with HCV undergo viral clearance while the remaining individuals develop a chronic infection which can lead to cirrhosis, hepatocellular carcinoma and the need for a liver transplant. Injection drug use is a major risk factor for HCV infections. This research studies the rate of HCV clearance in injection drug users from Southern China and the potential associated factors for clearance.

Research frontiers

This research studies HCV clearance in Chinese ethnicities with non-genotype 1 infections.

Innovations and breakthroughs

A low rate of HCV clearance was found in injection drug users of Chinese ethnicities. The majority of HCV infections were non-genotype 1 and many of the participants had current or previous co-infections with Hepatitis B viruses. Together these factors have previously been found to increase the level of viral clearance, suggesting other factors in the cohort are driving the low rate of viral clearance.

Applications

This research highlights the need for further studies of HCV infections in Chinese ethnicities which concentrate on the immunogenetics of the host.

Peer review

This paper describes the rate of hepatitis C viral RNA clearance in heroin users from south China and analysis of factors associated with it. The authors found a remarkably low rate of hepatitis C viral RNA clearance in the cohort and some possibly relating factors. The data presented are epidemiologically important, and the authors discussed the results appropriately.

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Effect of infliximab on small bowel stenoses in patients with Crohn's disease

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during maintenance therapy.

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Abstract

AIM: To assess prospectively small bowel stenoses in Crohn's disease (CD) patients treated with infliximab using Small Intestine Contrast Ultrasonography (SICUS).

METHODS: Twenty patients (M 12, age, 42.7 ± 11.8 years), 15 of whom showed obstructive symptoms indicating the presence of small bowel stenosis, and 5 without stenosis, were treated with infliximab (5 mg/kg at wk 0, 2, 6 and 5 mg/kg every 8 wk thereafter) for steroid refractoriness, fistulizing disease, or to avoid high-risk surgery. SICUS was performed at the induction phase and at regular time intervals during the follow-up period of 34.7 ± 16.1 mo (range 7-58). Small bowel stenoses were detected by SICUS, endoscopy and MRI.

RESULTS: In no case was progression of stenoses or the appearance of new ones seen. Of the 15 patients with stenosis, 5 stopped treatment after the induction phase (2 for no response, 3 for drug intolerance, one of whom showed complete regression of one stenosis). Among the remaining 10 patients, a complete regression of 8 stenoses (1 stenosis in 5 patients and 3 stenoses in one patient) was observed after 6-22 infliximab infusions.

CONCLUSION: In patients with CD treated with infliximab we observed: (a) No progression of small bowel stenosis and no appearance of new ones, (b) Complete regression of 1/22 stenosis after the induction phase and of 8/15 (53.3%) stenosis after 6-22 infusions

INTRODUCTION

The effect of infliximab on symptomatic Crohn's intestinal stenosis is controversial and, even though there is no direct evidence indicating anti-TNF α antibodies therapy enhances stricture formation, it is usual practice to withhold infliximab therapy in patients with intestinal stenosis. Indeed, two retrospective studies have reported intestinal obstructions as a possible adverse event^[1,2]. Such obstructive events have been interpreted as the outcome of an accelerated healing process that may trigger fibrosis within the inner layers of the gut wall. Conversely a review of large clinical studies concluded infliximab treatment did not increase the risk of developing strictures in patients with Crohn's disease (CD)^[3].

Because of the lack of non-invasive, radiation-free, techniques to assess transmural small bowel lesions, previous studies were retrospective and relied on assessments of the obstructive symptoms rather than of the stenotic lesion itself^[4-7]. Small intestine contrast ultrasonography (SICUS), performed after the ingestion of oral contrast, enables measurement of the wall thickness and luminal diameter of the small bowel^[8,9]. This technique can accurately assess the presence, size, and number of small bowel lesions in CD patients^[10,11].

In the present study, SICUS was used to assess the time course of small bowel stenosis in CD patients treated with infliximab, in a prospective follow-up investigation.

MATERIALS AND METHODS

Patients

As a part of a long-term prospective follow-up study, twenty patients (12 males and 8 females, age 42.7 ± 11.8 years) with CD of the small bowel received infliximab therapy because of the presence of fistulae (11 patients) and/or steroid dependence (7 patients) and/or azathioprine intolerance (5 patients) and/or extra-intestinal manifestation (1 patient with ankylosing spondylitis). Fifteen had small bowel stenoses and obstructive symptoms (6 had previously received extensive small bowel resections).

Diagnosis of CD was based on the criteria adopted in the EC-study^[12]. Medical history, including abdominal and extra-abdominal complaints, associated disease, CD behavior and CDAI at the first and last assessment of the follow-up, smoking status at diagnosis and at follow-up, family history, location of CD, duration of the disease, past surgery and endoscopic dilatation, and current and previous medical treatment were enquired and reported. Informed consent was obtained from each subject.

Protocol of the study

Patients were scheduled to receive an infusion of infliximab (5 mg/kg) at wk 0, 2 and 6 (induction phase) and 5 mg/kg every 8 wk thereafter (maintenance therapy). Each patient was prospectively evaluated with SICUS. SICUS was performed during the infliximab induction phase and at six-month intervals thereafter. Each patient was initially subjected to a standardized clinical interview and a physical examination, performed by one of two certified and experienced gastroenterologists (FB, EC). After an overnight fast, patients were consecutively submitted to SICUS and, on different days and in random order, to an endoscopic examination, with multiple mucosal biopsies, of the entire large bowel and terminal or neoterminal ileum. When deemed necessary additional investigations, including biochemistry, upper GI endoscopy, abdominal CT or MRI, were performed. The sonologist was aware of the diagnosis and clinical data, including bowel surgery, but was blinded to the results of endoscopy and of other investigations, and did not review the results of the previous SICUS examinations at each follow-up assessment.

At the end of the US investigation small bowel abnormalities were reported on a standardized form, with particular reference to presence, anatomical site and extension in centimeters of intestinal wall, and lumen alterations. Fistulas and abscesses were looked for and reported.

Small intestine contrast US (SICUS)

Real-time US was performed using Toshiba Tosbee (Tokyo, Japan) equipment with 3.5 MHz convex and 5 MHz linear array transducers. The sonologist (NP) had experience exceeding 7000 sonographic examinations of the whole abdomen and 4000 examinations of SICUS.

SICUS was performed according to a previously published^[8] method. Briefly, after the ingestion of 375 mL of macrogol contrast oral solution (Promefarm, Milan,

Italy) and after the contrast was seen to flow through the terminal ileum into the colon, a retrograde follow-through assessment of the entire small bowel was performed visualizing, in a caudo-cranial sequence, the contrast-filled ileal and jejunal loops. The body positions of patients were changed and abdominal compression with the US transducer was used whenever required to improve visualization of any single loop and detection of intestinal abnormalities after the ingestion of the oral contrast. All examinations were recorded on VHS to be re-examined at will.

Wall thickness and lumen diameter were measured at several sites (proximal, middle and distal) of the small bowel at the level of the maximally distended, and not contracting, intestinal loops.

The criteria for presence of CD ileal lesions were as follows^[10]: (1) Increased wall thickness (more than 3 mm) and lack and/or distortion of the intestinal folds; (2) presence of bowel stenosis defined as a lumen diameter of less than 1 cm, measured at the level of maximally distended loop, independent of the presence of pre-stenotic dilatation; and (3) bowel dilatation defined as lumen diameter more than 2.5 cm^[8,9]. The extension of the stenoses was expressed as the length of the segment with a lumen diameter of less than 1 cm^[8,9]. To distinguish a stenosis from a dynamic reversible reduction of luminal diameter due to intestinal contraction, multiple and prolonged, more than 15 min, observations of the narrowed tract were performed. Furthermore the presence of stenoses located in the terminal and neoterminal ileum were confirmed at endoscopy based on their inability to pass an 11 mm caliber endoscope; those in the more proximal small bowel segments were confirmed on at least two consecutive follow-up observations at SICUS and MRI.

For each detected lesion, site, number and length were reported on the record chart. Regression of intestinal stenosis was defined as an intestinal lumen with a diameter more than 1 cm as assessed by at least 2 follow-up SICUS evaluations, and confirmed at endoscopy for the lesions located in the terminal ileum, and at MRI for those located in the more proximal small bowel segment.

RESULTS

Induction phase

Fifteen patients had one or more stenosis of the small bowel; five patients did not have stenoses. In two female patients with penetrating CD behavior, infliximab therapy was discontinued for intolerance after the first i.v. administration. In an additional 2 female patients, one with entero-enteric fistulae and one with recto-vaginal fistula, treatment was discontinued after the induction period owing to a lack of response to the treatment. After the first 3 induction infusions there was a complete regression of one of the three upper GI stenoses in one male patient, who discontinued treatment owing to intolerance.

Maintenance phase

Fifteen patients then received maintenance therapy with

Table 1 Main characteristics of patients and small bowel lesions on maintenance infliximab therapy

Gender	Smoker	IB	DD (yr)	Age (yr) ¹	Previous surgery	Associated therapy	CDAI		FU months	Infusions ² number	Stenosis						
							Inclusion	Last			Site	Number	Length (cm)	Ø (mm)	WT (mm)	PSD	Regression ⁵
M	+	B3	11	24	3	-	74	13	26	11	PAI	1	10	5	5	-	8
M	-	B3	2.5	38	-	5-ASA	139.2	40.4	24	12	PI	1	20	7	10	+	8
M	+	B3	5.6	52	-	-	96	39.2	43	22	DI/TI	3	6	8	9	-	9
													3.5	9	6	-	22
													4.5	8	5	-	22
M	-	B2	21.5	22	1	-	28	28	54	27	PAI	1	7	9	8.5	-	6
M	+	B2	21	30	-	AZT/5-ASA	202	119	25	15	DI	1	5	4	6	+	15
F	+	B3	9	29	-	AZT/5-ASA	167	32	58	12	TI	1	5	5	7	+	12
M ⁴	+	B2	31	21	3	-	54	28	47	22 ³	PAI	1	7	7	6	+	-
M	-	B2	15.5	22	1	5-ASA	330.2	267.2	50	19	UGIT	4	3	4	9	+	-
													2	4	9	+	-
													6	4	9	+	-
													3	4	9	+	-
F	-	B3	30	18	3	5-ASA	241	12	51	4	PAI	1	15	9	7	+	-
M	+	B2	11	30	-	-	134.6	78.6	42	23	TI	1	25	7	9	+	-
M	-	B2	4.5	52	1	5-ASA	126	71.8	14	9							No stenosis
F	+	B3	3.2	26	-	5-ASA	154.6	0	38	9							No stenosis
M ⁴	-	B3	5.6	19	-	-	35.6	384	24	14							No stenosis
M ⁴	-	B3	15	38	-	5-ASA	306	144	7	6							No stenosis
F	+	B3	14.4	18	-	AZT	134	103	17	9							No stenosis

IB: Illness behavior; CDAI: CD activity index at inclusion and at the last follow-up assessment; DD: Disease duration; D: Distal ileum; F: Female; FU: Follow-up; M: Male; PAI: Pre-anastomotic ileum; PI: Proximal ileum; PSD: Prestenotic dilatation; TI: Terminal ileum; UGIT: Upper gastrointestinal tract; WT: Wall thickness; ¹Age at diagnosis; ²Some infusions were delayed for intercurrent events (e.g. pregnancy, infections *etc*); ³Strictureplasty after 6 infusions of infliximab, in maintenance therapy; ⁴Patients submitted to surgery during infliximab therapy; ⁵Number of infliximab infusions at regression.

infliximab. The main characteristics of patients receiving maintenance therapy are reported in Table 1 and Figure 1. During the follow-up period of 34.7 ± 16.1 mo (range 7-58 mo, median 38 mo), SICUS and, when required, endoscopic and imaging investigations, were performed at 10.7 ± 3.7 mo intervals in all but 3 patients who were assessed at induction and regularly subjected to follow-up starting at 21, 24 and 28 mo after the induction phase. Three patients were referred for surgery as they did not respond to maintenance therapy; one for entero-cutaneous fistulas after 3 infusions, one for severe recurrence after 12 infusions and one who previously received surgical operations and endoscopic dilatations for recurrent obstructive episodes after 6 infusions. Thirteen patients remained on maintenance therapy.

Patients with small bowel stenosis

Nine patients had 1 or more small bowel stenoses (1 patient with 4 jejunal stenoses; 1 patient with 1 stenosis at the level of proximal ileum; 1 patient with 3 stenoses at the level the distal and terminal ileum; 1 patient with 1 stenosis at the level of distal ileum; 2 patients with 1 stenosis at the level of terminal ileum; 3 patients with 1 stenosis at the level of neo-terminal ileum). Complete regression of 8 stenoses, 3 of which showed prestenotic dilatation, was observed in 6 patients; in 2 of these, the stenosis was at the level of the neo-terminal ileum. In one patient with three stenoses, the most proximal one regressed after 9 cycles of therapy, whereas the remaining two regressed after 22 cycles (Figure 2). Regression of all stenoses was confirmed in subsequent observations performed during the follow-up from a minimum of 1 mo to a maximum of 42 mo. In 3 patients, there was no variation in the pre-existing 6 stenoses, all with pre-stenotic dilatation, but the obstructive symptoms disappeared.

Patients without small bowel stenosis

Among the 5 patients without stenoses, none developed stenosis or obstructive symptoms and in one patient a regression of terminal ileum alteration was observed (Table 1). On average, CDAI improved during infliximab maintenance therapy, but no relationship was found between CDAI at time of induction or at last follow up observation and regression of stenoses.

DISCUSSION

The most relevant finding of the present study is the observation that during therapy with anti-TNF alpha antibodies there was no progression of pre-existing stenoses, no appearance of new ones, and complete regression of 1/22 (4.5%) small bowel stenoses after the induction phase and 8/15 (53.3%) small bowel stenoses after 6-22 maintenance infusions. In addition, despite the long duration of CD, no stenosis progressed to require surgery and in the presence of stenosis obstructive symptoms disappeared during infliximab treatment.

The limitations of this study are the small size of the population and the observational type of assessment. Confirmation in an *ad hoc* randomized controlled study performed in a larger sample size is required.

The strengths of this study are the prospective collection of data, the morphological assessment of CD stenosis with description of intestinal wall and lumen diameter and in comparison with previous reports, the relatively long duration of the follow-up in patients with small bowel stenosis treated with infliximab therapy.

A previously published review^[3] of prospectively collected data in the TREAT registry and from the ACCENT I trial concluded long-term treatment with infliximab was not associated with increased risk of

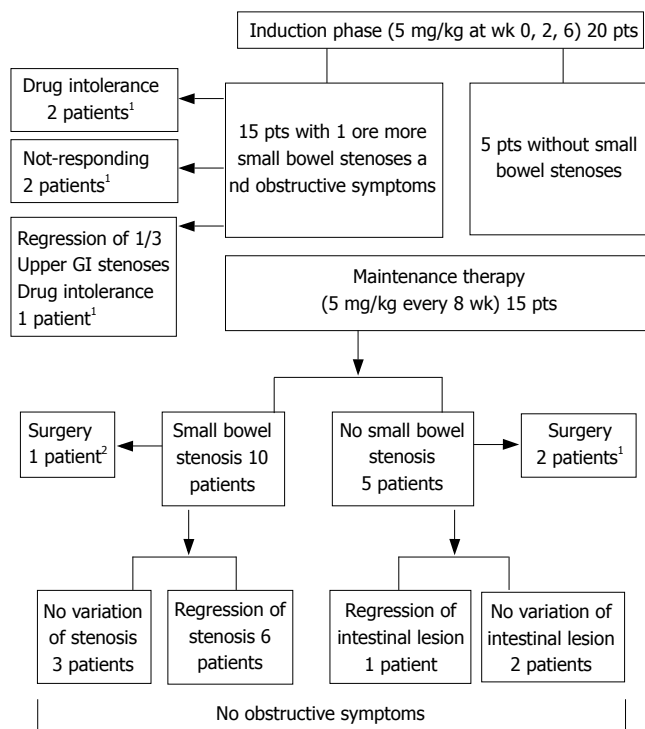


Figure 1 Table showing follow-up of patients on therapy with infliximab. ¹Stopped treatment; ²In maintenance treatment after surgery.

development of stenosis. These conclusions were based on clinical judgment, as radiological and endoscopic evaluations were performed at the discretion of the investigators, and not systematically in patients without obstructive symptoms. In addition, because patients with symptomatic stenosis were excluded from the study, the effect of infliximab on high-grade stenosis could not be assessed.

The present study refers to patients with CD of the small bowel treated with infliximab and belonging to a larger CD patient population assessed in a long-term prospective follow-up study, in which SICUS is used to evaluate the time course of small bowel lesions.

SICUS has enabled the normal values of wall thickness and luminal diameter of the small bowel and the reproducibility of measurements in healthy control subjects to be defined^[9]. Furthermore, the accuracy of SICUS in the assessment of the number, site and extension of small bowel lesions has been validated with intraoperative findings^[10].

In the present study, patients with symptomatic stenosis, some of whom have indications for surgery, and patients without stenosis, were prospectively followed up.

The observations obtained progressively in this case series indicate anti-TNF antibody therapy did not cause intestinal stenosis or obstructive symptoms. This finding is in contrast with previous reports of retrospective studies^[1,2], in which obstructive adverse events occurred after infliximab administration. However, retrospective analysis of these observations did not reveal whether the obstructive complication was due to a previously existing symptomatic stenosis and refractory to other therapies leading up to the use of infliximab. In the present study,

three patients received surgery during maintenance therapy. In one, who received stricturoplasty for stenosis, the lesion was already present and had surgical indications before infliximab treatment. Infliximab was administered in an attempt to avoid surgery in a patient with a previous history of intestinal resection and at risk of developing short bowel syndrome. After surgery, infliximab maintenance therapy was continued and after 2 years of follow-up no new stenosis has appeared and the patient shows no obstructive symptoms. In the second patient, who received surgery for enterocutaneous fistula, there was a temporary improvement after infliximab treatment and a symptomatic recurrence after the induction phase plus 3 cycles of maintenance therapy drug administration. In the third patient with entero-enteric fistulas, after an initial improvement of symptoms there was a severe recurrence after one year of maintenance therapy.

In three additional patients, despite the stenoses not changing after treatment, there was a disappearance of obstructive symptoms, and surgery was not required in the follow-up period. In the remaining six patients, there was complete regression of stenoses, irrespective of the CD type, whether stenosing or fistulizing, the site of the lesion, age of the patient, smoking status at follow-up, type and number of previous operations and pharmacological treatment, or the duration of CD.

It is reasonable to interpret the regression of the stenotic lesions as due to the anti-inflammatory effect of infliximab treatment.

A retrospective study evaluating the symptomatic response of obstructive symptoms for up to 18 months reported a favorable response in 9 of 11 patients treated with infliximab and concomitant immunosuppressive drugs^[13]. In the present study, only two patients of the 9 with stenoses receiving maintenance infliximab therapy received azathioprine concomitantly, indicating the favorable response was obtained after treatment with the biological agent, irrespective of the immunosuppressive drugs. In addition, regression of stenoses and obstructive symptoms was confirmed at all subsequent follow-up assessments. Such a favorable long-term effect could be a result of the maintenance treatment with infliximab that, when started immediately after endoscopic dilatation of small bowel stenosis, has been reported to prevent stenotic progression and obstructive complications in a 2-year follow up study^[14].

Tissue inflammation and fibrosis in the gut wall are regulated by cytokines with opposite effects. TNF has both a pro-inflammatory and anti-fibrotic action in the intestinal mucosa^[15]. Anti-TNF agents induce endoscopic^[16] and histological^[17] mucosal healing, but it is not known how they act in the deeper layers of the gut wall and whether, and how, they affect fibrosis at this level^[18]. The regression of stenotic lesions after administration indicates anti-TNF agents may act as antifibrotic agents in the deeper layers of the gut wall. It is also of relevance that 3 stenotic lesions with prestenotic dilatations regressed during infliximab treatment. A prestenotic dilatation is usually considered to be the result of a long-term stabilized and non-compliant luminal stricture due to circumferential fibrotic thickening of the gut wall. The intimate texture of this increased gut

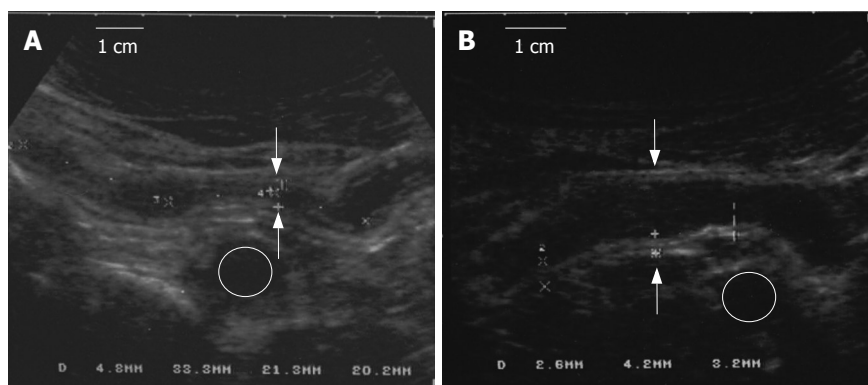


Figure 2 **A:** Before infliximab therapy: A stenotic lesion of the terminal ileum, with narrow lumen (white arrows) and increased thickness of the intestinal wall. The markers assess the length. The iliac vessel is indicated (circle); **B:** After infliximab therapy (22 cycles): The same ileal segment with an increased lumen diameter (12 mm) and reduced thickness of the intestinal wall. The iliac vessel is indicated (circle).

wall thickness cannot be ascertained with SICUS. It seems, however, that Infliximab treatment can remodel it^[18]. This event is possible because, in CD, fibrosis is mainly produced by mesenchymal cells, myocytes, interstitial cells of Cajal, and fibroblasts, all of which can proliferate and transform in fibrogenic cells or, under favorable conditions, redifferentiate into non-fibrogenic cells^[19-27]. Several pro- and anti-fibrogenic factors and intestinal fibroblasts participate in the development of intestinal fibrosis^[28] and should be antagonized to prevent fibrosis. In a murine model, chronic inflammation-induced intestinal fibrosis could be prevented by means of antisense NF- κ B^[29]. In the present study, it would appear that infliximab acts on the Crohn's lesion of the gut wall by either stopping or reversing the evolution of the stenotic process. However, in the patients investigated, no relevant clinical factors with high inter- and intra-individual variability predicted the response of the stenotic lesions to infliximab, indicating the degree of reversible inflammation and fibrosis may differ from one subject to another and may differ from one stenosis to another in the same subject.

Complete or partial regression of the stenotic lesions did occur early after induction, or late after several, up to 22, cycles of infliximab administration.

The great time and cycle response variability in the non-progression and regression of the stenoses may imply the effect of infliximab on the lesion may depend on at which stage of the CD fibrogenetic process infliximab is administered. Supportive of this hypothesis is the experimental evidence infliximab downregulates basic fibroblast growth factor and vascular endothelial growth factors involved in the process of intestinal fibrogenesis in CD^[30]. Theoretically, the earlier in the process of fibrogenesis infliximab is administered, the greater the probability to prevent stricture formation. Indeed, in two operated patients with early therapy after postsurgical recurrence there was a prompt regression of stenotic lesion, and in one of them, complete disappearance of pretreatment endoscopic and SICUS alterations were observed.

In conclusion, within the time-limit of the follow-up, it would appear infliximab is able to modify the expected time course of the disease in a relevant number of treated patients by stopping further development of, or causing regression of, stenotic lesions, thus being helpful to postpone or avoid surgical interventions.

Longer follow-up studies and properly structured

clinical trials are needed to assess whether infliximab loses its response over time and whether the potential risks of infliximab therapy outweigh the possible benefits.

COMMENTS

Background

Strictures are common features in Crohn's disease (CD). Several studies suggest that, in CD, fibrosis is mainly produced by mesenchymal cells, myocytes, interstitial cells of Cajal and fibroblasts, all of which can proliferate and transform into fibrogenic cells or, under favorable conditions, redifferentiate in non-fibrogenic cells. Anti-TNF agents induce mucosal healing. Concerns have been raised that rapid healing of narrowed segments induced by anti-TNF agents may further narrow the lumen. Several differing studies support the concept of an antifibrogenic role for infliximab, possibly down-regulating collagen production restoring migration and reducing collagen production of CD myofibroblasts.

Research frontiers

Follow-up studies based on the use of available imaging techniques to investigate, from different points of view, *in vivo*, the CD-involved intestinal wall and the response to different treatments.

Innovations and breakthroughs

The strengths of this study are: (1) The prospective collection of data; (2) the morphological assessment of CD stenosis; (3) the relatively long duration of the follow-up of patients with small bowel stenosis who had been treated with infliximab therapy; and (4) the study was not supported by any pharmaceutical company and the authors have no conflicts of interests.

Applications

These data suggest the presence of stenosis in patients with severe CD does not contraindicate anti-TNF treatment. If these data can be confirmed in an ad hoc randomized controlled study performed in a larger sample size, treatment with infliximab: (1) Could be indicated in patients with stenotic lesions requiring surgery; (2) may delay or even remove the need for surgery, and thus, favorably change the natural history of CD in patients with stenotic lesions of the small bowel.

Terminology

Small intestine contrast ultrasonography (SICUS) was not different from other structures (i.e bladder, gallbladder, stomach). Filling the small intestine with fluid enables visualization of the intestinal wall and lumen. Macrogol links to water that remains in the intestinal lumen. After the ingestion of 375 mL-500 mL of macrogol solution, it is possible to visualize the entire small intestine from the Treitz to the ileo-cecal valve.

Peer review

This is an interesting small series showing infliximab does not appear to have the major adverse effect on Crohn's strictures that was at one time feared.

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KIT exon 11 codon 557/558 deletion/insertion mutations define a subset of gastrointestinal stromal tumors with malignant potential

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Abstract

AIM: To study the association of the frequency and pattern of KIT and PDGFRA mutations and clinicopathological factors in a group of patients with gastrointestinal stromal tumors (GIST).

METHODS: Thirty patients with GIST were examined. Exons 9, 11, 13, and 17 of the KIT and exons 12 and 18 of the PDGFRA gene were analyzed for the presence of mutations by PCR amplification and direct sequencing.

RESULTS: KIT or PDGFRA mutations were detected in 21 of the 30 patients (70%). Sixteen patients had mutations within KIT exon 11, three within KIT exon 9, and two within PDGFRA exon 18. GISTs with KIT exon 9 mutations were predominantly located in the small intestine, showed a spindle cell phenotype, and were assessed as potentially malignant. GISTs with KIT exon 11 mutations were located in the stomach and intestine, showed mainly a spindle cell phenotype, and were scored as potentially malignant ($P < 0.05$). Tumors with KIT exon 11 codon 557/558 deletion/insertion mutations were found to be associated with a potentially malignant clinical behaviour ($P < 0.003$). GISTs with PDGFRA mutations located in stomach showed a mixed

cell phenotype and were classified as of very low or low moderate malignant potential.

CONCLUSION: Determination of KIT and PDGFRA mutations should be additional parameters for the better prediction of GISTs clinical behaviour. Tumors with deletion/insertion mutations affecting codons 557/558 of the KIT gene seem to represent a distinct subset of malignant GISTs.

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Key words: Gastrointestinal stromal tumors; KIT gene; Platelet derived growth factor receptor alpha; Mutations; Malignant

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INTRODUCTION

Gastrointestinal stromal tumors (GISTs) are the most common primary mesenchymal tumors of the gastrointestinal tract. Their biological behaviour is difficult to predict. GIST prognosis is largely dependent on the size, mitotic index, and presence or absence of metastases^[1-3]. We now know that GISTs may have either a well-developed or an incomplete myoid, neural, autonomic nerve, or mixed phenotype, or may remain undifferentiated^[2]. Typically, GISTs are immunohistochemical positive for KIT tyrosine kinase receptor which is perhaps their single best defining feature^[4,5].

Some GISTs are benign tumors and most of these are found incidentally. Other GISTs metastasize to liver or disseminate in the peritoneal cavity. Most of the latter type GISTs do not respond to chemotherapy and

ultimately kill the hosts. The pharmaceutical development and therapeutic implications of protein tyrosine kinase inhibitors has refocused the attention on GIST. Until now, the treatment with selective tyrosine kinase inhibitors, such as imatinib mesylate, for patients with GISTs has hinged on the KIT positive immunostaining tumors^[6]. Although the KIT positivity by immunohistochemistry becomes invaluable in the diagnosis of GISTs, some authors believe that a small subgroup of these tumors fulfill the clinical and morphological criteria of GISTs, and lack KIT expression^[7,8]. Studies in the last decade have established that activating mutations of KIT are present in 40% to 92% of GISTs and likely play an essential role in the development of these tumors^[9]. The subset of GISTs that lack detectable mutations could be divided into a group that has activating mutations in the related tyrosine kinase platelet-derived growth factor receptor alpha (PDGFRA) and a group without identified kinase mutations^[10,11].

A proportion of GISTs shows mutations in the regulatory juxtamembrane domain of the c-kit gene. These KIT mutations have been shown to represent gain-of-function mutations leading to ligand independent activation of the tyrosine kinase and the phosphorylation cascade that leads into mitogenic activation^[12,13]. Benign and malignant GISTs carry mutations in KIT and PDGFRA gene, but although these mutations vary among GISTs the definitive genotype/phenotype correlations are still under consideration^[9-11,14]. Moreover, currently it is not clear whether mutations are independent prognostic factors^[15-18].

In this study we examined a series of 30 patients with primary GISTs defined by different types of KIT and PDGFRA mutations, to investigate whether mutations' type and distribution are associated with GISTs clinical behaviour.

MATERIALS AND METHODS

Clinical and pathological data

Using the database of Surgery and Pathology Departments of Areteion University Hospital and Evangelismos General Hospital, we collected records with a pathologic diagnosis of stromal tumor within the GI tract. Thirty patients' records with the diagnosis of GIST between 2001 and 2005 were reviewed. Patients' age and gender, clinical manifestations, tumor size, pathological characteristics including cell type, cellularity, nuclear atypia, the number of mitoses, the presence of necrosis, or hemorrhage were recorded.

Formalin-fixed and paraffin-embedded samples were used for immunohistochemical examination. Tumors were divided according to their morphologic profile into four histological categories: epithelioid (Ep), spindle cell (Sp), mixed spindle cell with focal epithelioid component (mixed type 1), and tumors with mixed epithelioid and focal spindle cell component (mixed type 2). Mitoses were counted in 5 separate groups of 50 HPFs, a total area of 5 mm², and the highest of these 5 counts was recorded. Mitotic activity was scored as low (< 5/50 HPF), intermediate (5-10/50 HPF), or high (> 10/50 HPF). Tumors were as-

Table 1 Primer sequences used for KIT and PDGFRA PCR

Kit exon 9F ttggaaagctagtgggtca	Kit exon 9R atggtagacagagcctaaac
Kit exon11F ctattttccctttctccc	Kit exon11R tacccaaaagggtgacatgg
Kit exon 13F ctggacatcagtttgccag	Kit exon 13R aaaggcagctggacacggctta
Kit exon 17F ttctctccaacctaataag	Kit exon 17R cctttgcaggactgtcaagc
PDGFRA exon 12aF	PDGFRA exon 12aR
ccagttacctgtctctgtcat	tggaaactcccactcttgagtc
PDGFRA exon 12bF	PDGFRA exon 12bR
aaattcgctggagggtcatt	ggagggtaccccatggaagt
PDGFR exon 18F	PDGFRA exon 18R
agtgtgtccaccgtgatctg	gtgtgggaagtgtggaggta

signed to risk assessment categories based on size, mitotic index, and location according to published criteria^[17].

Immunohistochemistry

Immunohistochemical staining was performed using the following primary antibodies: KIT (CD 117 antigen, polyclonal, Dako, USA; 1:50 dilution), PDGFRA (polyclonal, Santa Cruz, California, USA, 1:400), a-smooth-muscle actin (clone asm-1, Dako; 1:200), desmin (clone DE-R-11, NovocastraLabs; 1:100), S-100 (clone S1/61/69, Novocastra Labs; 1:40), CD34 (clone QBEnd/10, Novocastra Labs; 1:50), Ki-67 (clone MM1, Novocastra Labs; 1:200) by a standard three step immunoperoxidase procedure (APAAP, DAKO, Glostrup, Denmark). Appropriate positive controls were run in parallel for all antibodies tested. According to the proportion of tumor cells showing an immunopositive reaction, tumors were classified as negative (< 10%) or positive (> 10%).

DNA sequencing

Exons 9, 11, 13, and 17 of the KIT and exons 12 and 18 of the PDGFRA gene were evaluated for the presence of mutations by PCR amplification and direct sequencing. DNA was extracted from formalin-fixed, paraffin embedded tissue samples using the PUREGEN DNA Purification System (Gentra Systems, Minneapolis, USA). The primer pairs used for PCR amplification and direct sequencing are given in Table 1.

Annealing temperature for the KIT exon 9 and 13 and for the PDGFRA primer sets was 53°C and for the KIT exon 13 and 17 primer sets 56°C, respectively. Amplification products were separated by 2% agarose ethidium bromide gel electrophoresis to confirm correct amplification. Products were purified with NucleoSpin Extract II kit (Macherey-Nagel, Duren, Germany) and applied to bi-directional sequencing on an ABI130 sequencer using the Big Dye Terminator v.1.1 KIT (Applied Biosystems, Foster City, CA).

Statistical analysis

Data were analyzed using Statistical software SPSS Version 13.0. Fisher's Exact Test of Independence was used to evaluate the statistical significance of associations in two-way contingency tables to determine whether KIT and PDGFRA gene mutations are independent of clinicopathological parameters. Statistical significance would be inferred at a two-tailed $P < 0.05$.

C-kit Exon 11 mutations																										
Case	550	551	552	553	554	555	556	557	558	559	560	561	562	563	564	565	566	567	568	569	570	577	578	579	580
	aaa	ccc	atg	tat	gaa	gta	cag	tgg	aag	gtt	gtt	gag	gag	ata	aat	gga	aac	aat	tat	gtt	tac		cct	tat	gat	cac
	K	P	M	Y	E	V	Q	W	K	V	V	E	E	I	N	G	N	N	Y	V	Y		P	Y	D	H
3 K	P	M	Y	E	V	Q	W	NP	V	V	E	E	I	N	G	N	N	Y	V	Y		P	Y	D	H	
4 K	P	M	Y	E	V	Q	W	NP	V	V	E	E	I	N	G	N	N	Y	V	Y		P	Y	D	H	
5 K	P	M	Y	E	V	Q			V	V	E	E	I	N	G	N	N	Y	V	Y		P	Y	D	H	
6 K	P	M	Y	E	V	Q			V	V	E	E	I	N	G	N	N	Y	V	Y		P	Y	D	H	
7 K	P	M	Y	E	V	Q	W	K	V	V	E	E	I	N	G	N	N	Y	V	Y		P	Y		H	
8 K	P	M	Y	E	V	Q	W	K	A	V	E	E	I	N	G	N	N	Y	V	Y		P	Y	D	H	
9 K	P	M	Y	E	V	Q	W	K	A	V	E	E	I	N	G	N	N	Y	V	Y		P	Y	D	H	
10 K	P	M	Y	E	V	Q			V	V	E	E	I	N	G	N	N	Y	V	Y		P	Y	D	H	
14 K							W	K	V	V	E	E	I	N	G	N	N	Y	V	Y		P	Y	D	H	
17 K	P	M	Y	E	V	Q	W	K	V	D	E	E	I	N	G	N	N	Y	V	Y		P	Y	D	H	
18 K					V	Q	W	K	V	V	E	E	I	N	G	N	N	Y	V	Y		P	Y	D	H	
19 K									V	D	E	E	I	N	G	N	N	Y	V	Y		P	Y	D	H	
22 K	P	M	Y	E	V	Q	W	K	V							N	N	Y	V	Y		P	Y	D	H	
23 K	P	M	Y	E	V	Q	W	K	V	V	E	E	I	N	G	N	N	Y	V	Y		P	Y	D	H	
27 K	P	M	Y	E	V	Q			V	V	E	E	I	N	G	N	N	Y	V	Y		P	Y	D	H	
28 K	Q								V	V	E	E	I	N	G	N	N	Y	V	Y		P	Y	D	H	

Figure 1 Kit exon 11 wild type and mutated amino acid sequences in 30 GISTs analyzed in this study.

RESULTS

Types and distribution of KIT and PDGFRA gene mutations among GISTs

All detected mutations, irrespective of whether involving single nucleotide substitutions or insertion/deletions, preserved the open reading frame. KIT or PDGFRA mutations were detected in 21/30 GIST patients (70%). Nine patients had no detectable mutations with the methods used. Sixteen patients had mutations within KIT exon 11 (Figure 1), three patients within KIT exon 9, and two patients within PDGFRA exon 18. No mutations were found in KIT exons 13, 17, or PDGFRA exon 12. The mutations within KIT exon 11 were heterogeneous and consisted of 10 simple deletions, 4 point mutations, and 2 insertions (Figures 1 and 2A). Codons 557 and 558 deletion/insertion mutations were found in 8 samples (50%) of the KIT exon 11 mutations followed by codon 560 (3 cases) and codon 559 (2 cases). Six of the mutations affecting codons 557/558 were deletions of various sizes and two were 3nt insertions.

KIT exon 9 mutations were all 6 bp insertions resulting in tandem duplication of the amino acids 502Ala-503Tyr (2 out of 3) (Figure 2B) or the amino acids 501Ser-502Ala. PDGFRA mutations affecting exon 18 consisted of a D842V substitution and a simple 12 bp deletion.

Clinicopathologic profile of patients with GISTs

The clinicopathological and molecular findings of the GIST patients are summarized in Table 2. Sixteen of the patients (53.3%) were male and 14 (46.7%) were female. Patients with KIT mutation exon 9 GISTs were exclusively male. Patient age ranged from 46 to 82 years with a median of 63.4 years. Sixteen tumors (53.3%) were located in the stomach, 9 (30%) in small intestine, and 5 (16.7%) in large intestine. Two out of three GISTs with KIT exon 9 mutations were located within the small intestine. GISTs with KIT exon11 mutations were located in the stomach

(6/16) and within the small and the large intestine (10/16). GISTs with PDGFRA mutations were exclusively located in the stomach (2/2). The majority of GISTs with no detectable mutations in KIT or PDGFRA (77.8%) were located in stomach.

Tumor size ranged from 0.7 cm to 19 cm (mean: 8, std: 5.2). The mean tumor size of GISTs with KIT exon 9 mutations was 6.7 cm (std: 8.1), and that of GISTs with KIT exon 11 mutations 7.8 cm (std: 8.2). The mean tumor's size of GISTs with KIT exon 11 codon 557/558 mutations was 11.3 cm, while 4.4 cm in GISTs without 557/558 mutations ($P = 0.025$) (Table 3). The mean size of GISTs with PDGFRA mutations was 11 cm (std: 7.8).

Grossly, most tumors were presented as circumscribed or lobulated masses. Cystic change was recognized in several cases. Histologically, the majority of tumors (66.7%) showed spindle cell phenotype, another 10% epithelioid, 13.3% mixed type 1, and 10% mixed type 2 phenotypes.

Deletions in KIT exon 11 were frequently found to be associated with a spindle cell phenotype, substitutions in KIT exon 11 were exclusively found to be associated with a mixed type 1 phenotype, while insertions in KIT exon 11 that affect codons 557/558 exclusively showed an epithelioid phenotype ($P < 0.05$) (Table 4). The majority of GISTs with KIT exon 9 mutations showed a spindle cell phenotype, whereas GISTs with PDGFRA exon 18 mutations were both of mixed type morphology. The mitotic count ranged from 0 to 22/50 HPF ($\times 400$) (mean: 4.8). The majority of tumors (70%) contained less than 5/50 HPF mitoses, 23.3% of tumors contained mitoses between 5 and 10/50 HPF, while only 6.7% of tumors contained mitoses $> 10/50$ HPF. GISTs with KIT exon 9 and exon 11 mutations exhibited a mean value of 5/50 HPF mitoses (std: 4.7) and 4.8/50 HPF mitoses (std: 4.7), respectively. In GISTs with PDGFRA mutations, mitotic activity was low with 2 to 4/50 HPF.

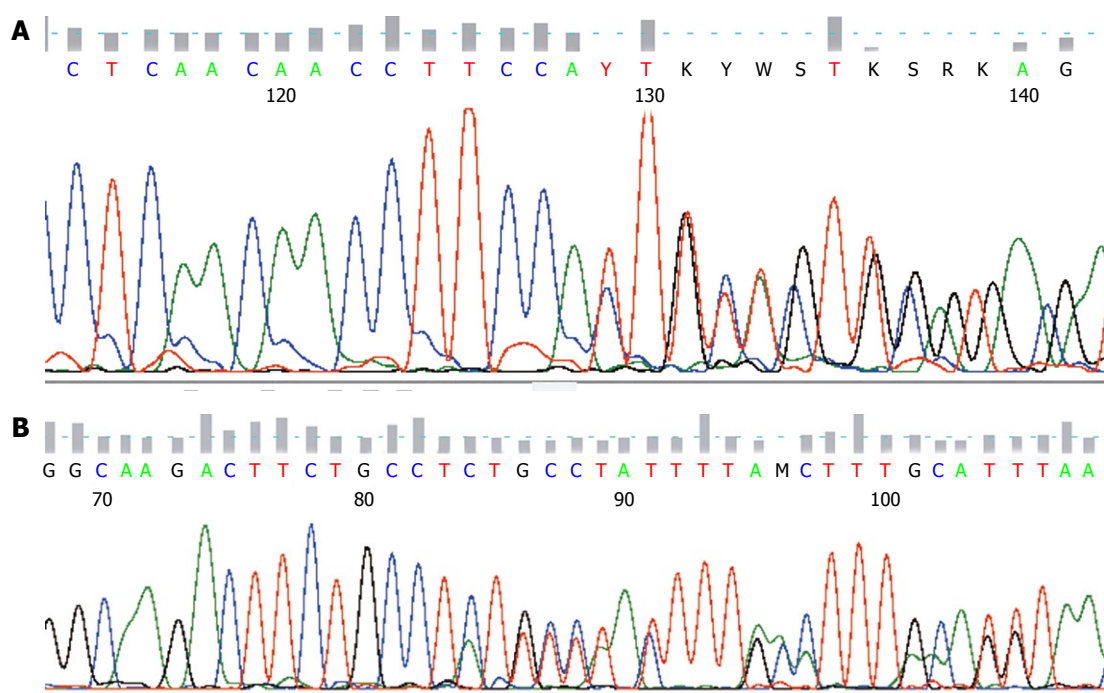


Figure 2 The sequencing data from a GIST. **A:** Showing KIT exon 11 deletion (p.P551-Q556del); **B:** Showing KIT exon 9 insertion (p.A502-Y503insAS).

Table 2 Clinicopathological and molecular characteristics of GIST patients

Case No.	Sex	Age	Site	Size (cm)	Mitoses (/50 HPF)	Differentiation	CD117	Diagnosis	KIT mutations	PDGFRA mutations
1	F	72	LI	9	6	SP	N	Malignant potential	wt	wt
2	M	65	SI	2.8	10	M1	P	Malignant potential	p.A502-Y503insA	wt
3	M	79	S	16	9	EP	P	Malignant potential	p.K558>NP	wt
4	F	60	SI	17	3	EP	P	Malignant potential	p.K558>NP	wt
5	F	59	SI	5	2	SP	P	Low malignant potential	p.W557-K558del	wt
6	M	72	SI	7.8	2	SP	P	Malignant potential	p.W557-K558del	wt
7	M	82	S	5.5	5	SP	P	Very low malignant potential	p.D579del	wt
8	M	52	SI	4	3	SP	P	Low malignant potential	p.V559A	wt
9	F	63	S	8	7	SP	P	Malignant potential	p.V559A	wt
10	F	70	SI	6	1	M1	P	Malignant potential	p.W557-K558del	wt
11	F	60	S	9	4	M1	P	Very low malignant potential	wt	p.D842V
12	M	61	S	13	2	M2	P	Low-moderate malignant potential	wt	p.I 843-D846del
13	M	64	S	9	3	SP	P	Very low malignant potential	wt	wt
14	F	70	S	1.5	1	SP	P	Benign	p.P551-Q556del	wt
15	M	66	S	10	20	SP	P	Malignant potential	wt	wt
16	M	60	SI	12	4	SP	P	Malignant potential	p.Y503-F504insAY	wt
17	F	62	SI	3.5	1	SP	P	Low malignant potential	p.V560D	wt
18	M	56	SI	4	1	SP	P	Low malignant potential	p.K550-E554del	wt
19	F	67	S	19	22	M1	P	Malignant potential	p.K550-K558del	wt
20	F	46	S	19	10	EP	P	Malignant potential	wt	wt
21	F	61	S	3.5	3	SP	P	Very low malignant potential	wt	wt
22	M	57	LI	8	5	SP	P	Malignant potential	p.V560D	wt
23	M	58	S	0.7	0	SP	P	Benign	p.V559-G565del	wt
24	M	60	S	7.5	3	M2	P	Very low malignant potential	wt	wt
25	M	67	S	8	1	M2	P	Very low malignant potential	wt	wt
26	M	72	S	5.5	1	SP	P	Very low malignant potential	p.Y503-F504insAY	wt
27	F	70	LI	17	9	SP	P	Malignant potential	p.W557-K558del	wt
28	M	61	LI	2.6	6	SP	P	Malignant potential	p.P551-K558del	wt
29	F	53	S	5	0	SP	N	Very low malignant potential	wt	wt
30	F	57	LI	2.5	0	SP	P	Low malignant potential	wt	wt

S: Stomach; SI: Small intestine; LI: Large intestine; SP: Spindle cell; EP: Epithelioid; M1: Mixed type 1; M2: Mixed type 2; P: Positive; N: Negative.

Cellularity was high in 40% of the tumors, moderate in 36.7%, and mild in 23.3%. Cellularity was high in the majority (86%) of GISTs with KIT exon 9 mutations,

moderate in the majority GISTs with exon 11 mutations, and moderate or high in all GISTs with PDGFRA mutations. The majority (73%) of the tumors showed

Table 3 Clinicopathological data of GIST patients according to the presence of codon 557/558 deletion/insertion mutations

Variable	Wt (%)	557/558 mutations (%)	Non 557/558 mutations (%)	P
Age (yr)	61.7	67.2	62.5	NS
Size (cm)	8.2	11.3	4.4	0.025
Mitoses				
Low (\leq 5/50 HPF)	71.4	50	87.5	NS
Intermediate 5-10/50 HPF	21.4	37.5	12.5	
High ($>$ 10/50 HPF)	7.1	12.5		
Differentiation				
Epithelioid	7.1	25		NS
Spindle cell	57.1	50	100	
Mixed type 1	14.3	25		
Mixed type 2	21.4			
Risk assessment				
Benign			25	0.003
Very low malignant potential	57.1		12.5	
Low malignant potential	7.1	12.5	37.5	
Low moderate malignant potential	14.3			
Malignant potential	21.4	87.5	25	

no necrosis. Absence of necrosis was present in 2/3 of GISTs with KIT exon 9-11 mutations, and in all GIST patients with PDGFRA mutations. A high proportion of GISTs with codon 557/558 mutations (50%) was found to be necrotic. The majority (66.6%) of the tumors showed no hemorrhage. Hemorrhage was found in all cases of GISTs with PDGFRA mutations ($P = 0.038$), but to be absent in most of the GISTs with KIT mutations. The majority (80%) of the tumors showed no ulceration. All the tumors with ulceration were positive for KIT exon 9 or 11 mutations.

Immunohistochemically, 28 tumors were positive for KIT expression. All GISTs with KIT and PDGFRA mutations showed weak to strongly and diffuse positive staining for the KIT gene. PDGFRA expression was immunohistochemically detected in 18 cases (60%). Alpha-smooth muscle actin, desmin, and S-100 protein were positive in 12 (40%), 3 (10%), and 4 (13.3%) cases, respectively. A-SMA was positive in 47.6% of GISTs with KIT mutations. CD34 expression was positive in 20 (67%) cases. Ki-67 expression was strongly positive (5%) in 13 cases (43%). Applying the clinical behaviour (risk assessment) of primary tumors according to Miettinen and Lasota (2006) 6.7% of GISTs were benign, 30% of very low malignant, 16.7% of low malignant, 6.7% of low moderate malignant, and 40% of malignant potential. 57.9% of GISTs with KIT mutations were associated with malignant potential ($P = 0.003$) whereas none of the GISTs with PDGFRA mutations was assessed as of malignant potential. 66.7% of the GISTs with exon 9 KIT mutations and 56.3% of those with exon 11 KIT mutations were assessed as of malignant potential ($P = 0.036$) (Tables 4 and 5). Tumors with KIT exon 11 codon 557/558 mutations showed a statistically significant correlation with malignant potential scoring (87.5% *vs* 25% of the non 557/558, $P = 0.003$).

Table 4 GIST phenotypes according to the presence and type of KIT mutations

	Differentiation				P
	Epithelioid (%)	Spindle cells (%)	Mixed type 1 (%)	Mixed type 2 (%)	
KIT mutation					
Positive	10.5	73.3	15.8		NS
Negative	9.1	54.5	9.1	27.3	
Exon 9		66.7	33.3		NS
Exon 11	12.5	75	12.5		
Insertions	100				0.03
Deletions		80.0	20.0		
Substitutions			100		

Table 5 Risk assessment according to the KIT/PDGFRA mutations

	Risk assessment					P
	Benign (%)	Very low malignant potential (%)	Low malignant potential (%)	Low moderate malignant potential (%)	Malignant (%)	
KIT mutation						
Positive	10.5	10.5	21.1		57.9	0.003
Negative		63.6	9.1	18.2	9.1	
Exon 9		33.3			66.7	0.036
Exon 11	12.5	6.3	25		56.3	
PDGFRA mutation						
Positive						NS
Negative		50		50		

DISCUSSION

In the late 1990s it was shown that GISTs share morphological, immunohistochemical, and genetic characteristics with the interstitial cells of Cajal (ICCs). Most GISTs express strongly and specific the tyrosine kinase KIT oncoprotein that it was claimed to be required for the diagnosis^[4,5]. It is now clear that a small but significant group of GISTs (5%-10%) are KIT negative^[7,8]. It seems that GISTs probably do not constitute a homogenous group of tumors. Until now the prediction of their biological behaviour depended on classic clinicopathological characteristics as size and mitotic activity or location. Moreover, in the last years a significant correlation has been suggested between these pathological parameters and molecular alterations^[11].

It has been suggested that tumors' location were associated with mutations. GISTs with KIT exon 9 mutations were predominantly located in the small intestine whereas GISTs with PDGFRA mutations represent gastric tumors^[10,15,17]. Other studies failed to find a significant association between KIT exon 11 mutation status and tumor location^[18,19].

With regard to the primary tumor location, our results indicated that KIT exon 9 mutations were almost always detected in GISTs of intestinal origin, whereas PDGFRA mutations were associated with GISTs of gastric origin. The incidence of exon 11 KIT mutations did not appear to be related to tumor location.

The mutation type in GISTs has been reported to

be associated with the phenotype^[20,21]. With respect to histological phenotype, KIT exon 11 mutations were strongly associated with spindle cell phenotype GISTs. Deletions in KIT exon 11 have been mostly associated with spindle cell phenotype, substitutions in KIT exon 11 were associated exclusively with mixed cell phenotype, whereas insertions in KIT exon 11 affecting codons 557/558 were exclusively associated with epithelioid cell phenotype GISTs. PDGFRA mutations, in this study, were exclusively associated with mixed type phenotype and low risk assessment GISTs.

An association between the occurrence of KIT exon 9 and 11 mutations in GISTs and malignancy was suggested by previous studies^[14,22]. Our data support this notion by showing a significant association between KIT exon 11 and exon 9 mutations and malignant GISTs. Moreover, in our data this association remains significant when only the exon 11 mutations affecting codons 557/558 were analysed. This observation is in agreement with previous studies that indicate an association of 557/558 deletions with poor prognosis and metastatic behaviour^[23]. In addition to deletions it is possible that insertions affecting the 557/558 codon, although rare, are also associated with malignant phenotype as suggested by previously published data^[24-26]. Codons 557/558 have been also found to represent significant a/a residues either for inhibitory role in the control of the receptor tyrosine kinase activity (Tryp557) or in constitutive receptor phosphorylation (Lys558)^[27,28].

In conclusion, in this study we have analyzed the frequency and pattern of KIT and PDGFRA mutations in a group of GISTs, and we presented evidence that tumors defined by KIT codon 557/558 deletion/insertion mutations represent a subgroup of GISTs with malignant clinical behaviour. These findings underline the need for a new classification system that would integrate specific molecular alterations to the pathological criteria.

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COMMENTS

Background

In the late 1990s it was shown that most gastrointestinal stromal tumors (GISTs) share morphological, immunohistochemical, and genetic characteristics with the interstitial cells of Cajal (ICCs). Most GISTs express strongly and specifically the tyrosine kinase KIT oncoprotein that it was claimed to be required for the diagnosis. GISTs are the most common primary mesenchymal tumors of the gastrointestinal tract. Their biological behaviour is difficult to predict. GISTs prognosis is largely dependent on the size, mitotic index, and presence or absence of metastases. We now know that GISTs may have either a well-developed or an incomplete myoid, neural, autonomic nerve, or mixed phenotype, or may remain undifferentiated. Typically, GISTs are immunohistochemical positive for KIT tyrosine kinase receptor which is perhaps their single best defining feature. Studies in the last decade have established that activating mutations of KIT are present in 40% to 92% of GISTs and likely play an essential role in the development of these tumors. The subset of GISTs that lack detectable mutations could be divided into a group that has activating mutations in the related tyrosine kinase platelet-derived growth factor receptor alpha (PDGFRA) and a group without identified kinase mutations. A proportion of GISTs shows mutations in the regulatory juxtamembrane domain

of the c-kit gene. These KIT mutations have been shown to represent gain-of-function mutations leading to ligand independent activation of the tyrosine kinase and the phosphorylation cascade that leads into mitogenic activation.

Research frontiers

Benign and malignant GISTs carry mutations in KIT and PDGFRA gene, but although these mutations vary among GISTs the definitive genotype/phenotype correlations are still under consideration. Moreover, currently it is not clear whether mutations are independent prognostic factors. It has been suggested that tumors' location was associated with mutations in GISTs. KIT-MT exon 9 GISTs were predominantly located in small intestine whereas GISTs with PDGFRA mutations represent gastric tumors. Others studies failed to find a significant association between KIT exon 11 mutation status and tumor location. The mutation type in GISTs has been reported to be associated with the phenotype. With respect to histological phenotype, KIT exon 11 mutations were strongly associated with spindle cell phenotype GISTs. Deletions in KIT exon 11 have been mostly associated with spindle cell phenotype, substitutions in KIT exon 11 were associated exclusively with mixed cell phenotype, whereas insertions in KIT exon 11 affecting codons 557/558 were exclusively associated with epithelioid cell phenotype GISTs.

Innovations and breakthroughs

In addition to deletions it is possible that insertions affecting the 557/558 codon, although rare, are also associated with malignant phenotype as suggested by previously published data. Codons 557/558 have been also found to represent significant a/a residues either for inhibitory role in the control of the receptor tyrosine kinase activity (Tryp557) or in constitutive receptor phosphorylation (Lys558). We presented evidence that tumors defined by KIT codon 557/558 deletion/insertion mutations represent a subgroup of GISTs with malignant clinical behaviour.

Applications

These findings underline the need for a new classification system that would integrate specific molecular alterations to the pathological criteria.

Peer review

This is a nice study which analysed the frequency and pattern of KIT and PDGFRA mutations in a group of patients with GISTs and the association of these mutations with other clinicopathological factors.

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RAPID COMMUNICATION

Portal hemodynamics as predictors of high risk esophageal varices in cirrhotic patients

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compensated cirrhotic patients: Portal hypertensive index > 2.08 and spleen size > 15.05 cm. These factors may help identifying patients with a low probability of LEV who may not need upper gastrointestinal endoscopy.

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Key words: Liver cirrhosis; Doppler ultrasound; Portal hemodynamics; Esophageal varices; Prediction

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Abstract

AIM: To evaluate portal hypertension parameters in liver cirrhosis patients with and without esophageal varices (EV).

METHODS: A cohort of patients with biopsy confirmed liver cirrhosis was investigated endoscopically and with color Doppler ultrasonography as a possible non-invasive predictive tool. The relationship between portal hemodynamics and the presence and size of EV was evaluated using uni- and multivariate approaches.

RESULTS: Eighty five consecutive cirrhotic patients (43 men and 42 women) were enrolled. Mean age (\pm SD) was 47.5 (\pm 15.9). Portal vein diameter (13.88 ± 2.42 vs 12.00 ± 1.69 , $P < 0.0005$) and liver vascular index (8.31 ± 2.72 vs 17.8 ± 6.28 , $P < 0.0005$) were found to be significantly higher in patients with EV irrespective of size and in patients with large varices (14.54 ± 1.48 vs 13.24 ± 2.55 , $P < 0.05$ and 6.45 ± 2.78 vs 10.96 ± 5.05 , $P < 0.0005$, respectively), while portal vein flow velocity (13.25 ± 3.66 vs 20.25 ± 5.05 , $P < 0.0005$), congestion index (CI) (0.11 ± 0.03 vs 0.06 ± 0.03 , $P < 0.0005$), portal hypertensive index (2.62 ± 0.79 vs 1.33 ± 0.53 , $P < 0.0005$), and hepatic (0.73 ± 0.07 vs 0.66 ± 0.07 , $P < 0.001$) and splenic artery resistance index (RI) (0.73 ± 0.06 vs 0.62 ± 0.08 , $P < 0.0005$) were significantly lower. A logistic regression model confirmed spleen size ($P = 0.002$, AUC 0.72) and portal hypertensive index ($P = 0.040$, AUC 0.79) as independent predictors for the occurrence of large esophageal varices (LEV).

CONCLUSION: Our data suggest two independent situations for beginning endoscopic evaluation of

Tarzamni MK, Somi MH, Farhang S, Jalilvand M. Portal hemodynamics as predictors of high risk esophageal varices in cirrhotic patients. *World J Gastroenterol* 2008; 14(12): 1898-1902 Available from: URL: <http://www.wjgnet.com/1007-9327/14/1898.asp> DOI: <http://dx.doi.org/10.3748/wjg.14.1898>

INTRODUCTION

The most common clinical manifestations of portal hypertension in patients with liver cirrhosis are esophageal varices (EV). Bleeding EV are of the most apprehensive complications of portal hypertension contributing to the estimated 32000 deaths annually attributed to cirrhosis^[1]. Reducing morbidity and mortality of EV remains a challenge for physicians managing patients with chronic liver disease.

The incidence of EV in patients with cirrhosis ranges from 35% to 80%. Approximately one third of the patients with EV experience variceal bleeding, which in up to 70% of the survivors is followed by repeated bleeding episodes^[2]. Esophageal variceal bleeding might be a deadly complication in liver cirrhosis patients with portal hypertension^[3,4]. A screening is indicated in patients with newly diagnosed cirrhosis. Medical treatment must be considered as soon as varices are detected to prevent a first bleeding^[5].

It has been shown that the risk of EV bleeding is related to its size^[6]. Large esophageal varices (LEV) are at a greater risk, which is possibly due to a higher variceal wall tension^[7]. Availability of non-invasive methods for detection of LEV may help limit the number of endoscopic procedures.

The estimation of blood flow volume with Doppler sonography is non-invasive and allows physiologic measurements that were impossible to obtain in the past. It was widely used to explore the relationship between EV hemodynamics associated with portal hypertension and liver cirrhosis^[8,9]. Main characteristics of portal hypertension like a decrease in portal flow velocity or an increase in portal vein diameter are detectable by this means^[10,11]. However, no consistent alternative has been reported to replace endoscopic assessment of such patients through time yet. In this study, we investigated the hemodynamic features of the portal vein in two groups of patients with liver cirrhosis, namely those with and those without EV, as well as considering large varices with an advanced risk of bleeding.

MATERIALS AND METHODS

Consecutive newly diagnosed cirrhotic patients who were visited at our institute participated in a prospective study from May 2006 to August 2007 prior to treatment. The diagnosis of cirrhosis was based on a liver biopsy evaluation. Patients on diuretic or vasoactive treatment, with previous gastrointestinal bleeding, hepatorenal syndrome during the past 3 mo, evidence of portal vein thrombosis on ultrasonography, and patients with clear signs of portal hypertension (ascites, porto-systemic shunts or hepatic encephalopathy) were excluded.

All patients underwent endoscopy after color Doppler-ultrasonic examination by the same gastroenterologist blinded to the results of duplex Doppler. They were evaluated for the presence and grade of EV, the presence of gastric varices, and portal hypertensive gastropathy (PHG). In the presence of EV, size was graded as I-IV using the Paquet grading system^[12]. Moreover, patients were classified either as having LEV (grade III-IV) or not (no varices or grade I - II).

All patients were kept fasting overnight prior to the procedure at our institution. They were examined in the supine position during quiet respiration. The following main Doppler factors were always taken by the same equipment (with a 3.5- MHz linear - array transducer, EUB-525 Hitachi) and by the same operator ($k = 0.80$): (1) Portal vein flow velocity as time average maximal velocity in cm/s and portal vein diameter^[13]; (2) hepatic artery resistance index (RI) measured in the intrahepatic main branches^[14] [$RI = (\text{systolic velocity} - \text{end diastolic velocity})/\text{systolic velocity}$]; (3) splenic artery RI measured intraparenchymally near to hilum^[15]; (4) spleen size (length of its longest axis); and (5) presence of portal-systemic collaterals.

The following indices were calculated: (1) The liver vascular index as the ratio of portal venous velocity to hepatic arterial pulsatility index; (2) congestion index (CI) of the portal vein with dividing portal vein cross-sectional area by portal blood velocity^[16]; and (3) portal hypertensive index as $(\text{hepatic artery RI} \times 0.69) \times (\text{splenic artery RI} \times 0.87) / \text{portal vein mean velocity}$.

Data were analyzed with SPSS for windows version 13. Descriptive statistics including means, standard deviations, and frequencies were computed. The chi square test was used to compare differences, and student's *t* test was

Table 1 Baseline characteristics of 85 cirrhotic patients [as *n* (%) or mean \pm SD]

Gender	
Male	43 (50.6)
Female	42 (49.4)
Etiology	
Hepatitis B virus (HBV)	40 (47.0)
Hepatitis C virus (HCV)	12 (14.1)
Cryptogenic	14 (16.5)
Autoimmune hepatitis (AIH)	17 (20.0)
Alcohol	2 (2.4)
Wilson's disease	1 (1.2)
Size of esophageal varices	
None	16 (18.8)
Small (grade I-II)	50 (58.8)
Large (grade III-IV)	19 (22.3)
Size	7.9 (\pm 3.4)
Gastric varices	11 (12.9)
Portal hypertrophic gastropathy	75 (88.2)
Portal vein diameter (mm)	13.5 (\pm 2.4)
Splenic axis (cm)	15.7 (\pm 3.1)
Portal vein flow (cm/s)	14.6 (\pm 4.8)
Splenic artery resistance	0.7 (\pm 0.1)
Hepatic artery resistance	0.7 (\pm 0.1)

used to compare means of variables. Values were considered significant if $P < 0.05$ (95% CI). A logistic regression equation was developed to predict presence and grade of EV. The sensitivity and specificity of the prediction rule were estimated by means of a receiver operating characteristic (ROC) curve and area under the curve (AUC) was reported for independent predictors.

RESULTS

Eighty five consecutive cirrhotic patients (43 men, 42 women) were enrolled in the study. Mean age (\pm SD) of the study population was 47.5 (\pm 15.9) years. Table 1 shows the patients' baseline characteristics. Hepatitis B virus (HBV) infection was the only cause of cirrhosis in most of our patients.

Thirteen patients had EV grade 1, 37 grade 2, and 19 grade 3. Gastric varices were detected in 11 patients (ten type 1 and one type 2).

Univariate analysis showed that most of the echo-Doppler parameters were related to presence of EV (Table 2). Portal vein flow velocity and liver vascular index was significantly higher in patients with EV while they had lower portal vein diameter, CI, portal hypertensive index, and hepatic and splenic artery RI. Presence of LEV was related to all of the echo-Doppler parameters described in Table 3.

Portal hypertensive index ($P = 0.002$) and congestive index ($P = 0.002$) were significantly higher, and portal vein flow velocity ($P < 0.0005$) and liver vascular index ($P \leq 0.0005$) were significantly lower in patients with PHG. Liver vascular index was independently correlated with PHG ($P = 0.018$). Portal PHG was present in 94.2% of the patients with EV ($P = 0.002$) and in all of the patients with gastric varices.

A logistic regression model showed that the parameters were not a good predictor of the presence of esophageal or gastric varices. However, spleen size and portal hyper-

Table 2 mean \pm SD of the primary and derivative echo-Doppler factors in study population according to presence of esophageal varices in any size

	With EV	Without EV	P value	AUC
Portal vein flow velocity (cm/s)	13.25 \pm 3.66	20.25 \pm 5.05	< 0.0005	0.113
Portal vein diameter (mm)	13.88 \pm 2.42	12.00 \pm 1.69	0.004	0.242
Hepatic artery resistance index	0.73 \pm 0.07	0.66 \pm 0.07	0.001	0.210
Splenic artery resistance index	0.73 \pm 0.06	0.62 \pm 0.08	< 0.0005	0.168
Spleen size (cm)	15.98 \pm 3.01	14.76 \pm 3.66	0.166	0.431
Liver vascular index	8.31 \pm 2.72	17.08 \pm 6.28	< 0.0005	0.114
Congestion index	0.11 \pm 0.03	0.06 \pm 0.03	< 0.0005	0.128
Portal hypertensive index	2.62 \pm 0.79	1.33 \pm 0.53	< 0.0005	0.072

EV: Esophageal varices; AUC: Area under the curve.

tensive index were reported as predictors of LEV as presented in Table 4. We examined threshold values for these independent predictors of LEV for achieving a sensitivity > 75%. Portal hypertensive index > 2.08 and spleen size > 15.05 cm reached a sensitivity of 79% for detecting LEV.

DISCUSSION

Variceal gastrointestinal bleeding is one of the most common life-threatening complications of portal hypertension with significant morbidity and mortality. Variceal size is identified to be one of the most important factors responsible for first variceal hemorrhage^[17]. 10% to 20% of small varices progress in size during one year^[18] which is close to 20% to 30% risk of bleeding in first 2-year after first detection^[19]. It seems that recognizing patients with elevated risk of bleeding for on time interventions will reduce morbidity and cost in initial diagnosis or periodic intervals thereafter.

Consensus based guidelines recommend endoscopic screening of all cirrhotic patients for the presence of varices at the time of diagnosis^[20]. Relatively low risk of bleeding in compensated cirrhotic patients and a need to avoid invasive and avoidable procedures, suggests performing an upper gastrointestinal endoscopy only on those patients with clinical evidence of portal hypertension^[21].

Even though, the available data are insufficient to determine a reliable non-invasive predictive tool to categorize cirrhotic patients along with significant risk for bleeding. Researchers have designed studies based on clinical, biochemical, and radiographic measurements as to when one should begin endoscopic screening for the presence of EV with cirrhosis. Such attempts have been made to identify non-invasive procedures for either reducing or eliminating the need for screening endoscopy. Researchers support non-invasive methods (duplex Doppler sonography) in measurement of functional hepatic flow in cirrhotic patients, which can estimate hepatic reserve function^[22].

Our study, based on information achieved from newly diagnosed compensated liver cirrhosis patients demonstrated a correlation of portal hemodynamics with the presence of LEV and with a higher diagnostic accuracy with LEV on univariate analysis. However, on multivariate analysis, only increased spleen size and portal hypertensive index were found to have an independent predictive value

Table 3 mean \pm SD of the primary and derivative echo-Doppler factors according to presence of large esophageal varices

	With LEV	Without LEV	P value	AUC
Portal vein flow velocity (cm/s)	12.13 \pm 2.59	15.26 \pm 5.06	0.001	0.715
Portal vein diameter (mm)	14.54 \pm 1.48	13.24 \pm 2.55	0.037	0.748
Hepatic artery resistance index	0.80 \pm 0.06	0.70 \pm 0.06	0.003	0.820
Splenic artery resistance index	0.76 \pm 0.11	0.69 \pm 0.06	< 0.0005	0.656
Spleen size (cm)	17.62 \pm 3.05	15.21 \pm 2.99	0.003	0.724
Liver vascular index	6.48 \pm 2.78	10.96 \pm 5.05	< 0.0005	0.817
Congestion index	0.14 \pm 0.04	0.09 \pm 0.03	< 0.0005	0.797
Portal hypertensive index	3.18 \pm 0.90	2.14 \pm 0.77	< 0.0005	0.791

LEV: Large esophageal varices; AUC: Area under the curve.

Table 4 Logistic regression model to predict the presence of large esophageal varices in newly diagnosed patients with compensated cirrhosis

	P value	Odds ratio	95% CI
Congestive index	0.252	0	-
Portal hypertensive index	0.040	4.83	1.08-21.65
Liver vascular index	0.151	0.72	-
Spleen size	0.002	1.77	1.23-2.55

which has been the most consistently identified predictors in previous studies. Our data suggests two independent situations for beginning endoscopic evaluation of compensated cirrhotic patients: Portal hypertensive index > 2.08 and spleen size > 15.05 cm; restraining the need for upper gastrointestinal endoscopy of compensated cirrhosis.

It may be explained according to the issue that palpable spleen as well as LEV may both be related to the presence of a higher portal pressure. Different factors found to be important for this purpose included splenomegaly^[23-28], thrombocytopenia^[23-30], ascites^[25,27], hepatic encephalopathy^[25], serum albumin concentration^[30], serum bilirubin levels^[30], and Child-Pugh score^[27,28]. Thus, the results of our study are consistent with those of the previously published data.

Echo-Doppler parameters like splenic artery RI and portal hypertensive index have been reported to have a specificity > 70% (for most thresholds) when comparing portal hypertensive patients with CLD patients without clinically relevant portal hypertension^[8].

Esophagogastric varices exactly reflect the presence of portal hypertension. But the correlation between esophagogastric varices and PHG is obscure^[31]. Our study revealed a correlation between EV and the presence of gastropathy, and all of the patients with LEV had gastropathy.

Our study group represented a selected group of patients with liver cirrhosis attending a tertiary care center, but criteria for excluded patients (clear signs of portal hypertension) and preferring patients without history of GI bleeding achieved a better sample. Results would be best applied in patients attending large hospitals and further studies will be necessary regarding this aspect. Such studies may be particularly indicated because of

differences in the etiology of liver disease in dissimilar populations. The most common etiologies of cirrhosis in our population are either cryptogenic or HBV infection^[32].

Our data indicate that using non-invasive tools for estimating spleen size and portal hypertensive index allows predicting the presence of LEV with a fairly high accuracy. Values for the non-invasive indicators from this study and comparables need to be validated in a prospective study. Selecting patients for an upper GI endoscopy may be cost effective and, on the other hand, will define patients who need a critical management.

COMMENTS

Background

Bleeding esophageal varices (EV) are of the most apprehensive complications of portal hypertension in patients with liver cirrhosis. EV bleeding is a potentially deadly complication in such patients and is considered as an indication for screening in patients with newly diagnosed cirrhosis.

Research frontiers

Availability of non-invasive methods may help limit the number of endoscopic procedures performed for detection of large esophageal varices (LEV) which hold the higher risk for bleeding.

Related publications

Researchers have mentioned relations between portal hemodynamic situation and risk of EV or bleeding of them but available data are still insufficient to determine a reliable non-invasive predictive tool to categorize cirrhotic patients along with significant risk for bleeding.

Innovations and breakthroughs

This study evaluates newly diagnosed patients with no complications who may benefit from non-invasive procedures. Etiology of liver cirrhosis in our study population is different from Western community.

Applications

Using non-invasive tools for estimating spleen size and portal hypertensive index makes it possible to predict the presence of LEV. These values should ultimately be validated in a prospective study before being used to determine which patients should undergo esophageal variceal screening endoscopy.

Terminology

Size of the spleen and portal hypertensive index are measured by ultrasonography. Portal hypertensive index in details is (Hepatic artery RI*0.69)/(splenic artery RI*0.87)/portal vein mean velocity.

Peer review

It is a nice study to evaluate and compare the differences in the parameters of portal hypertension in liver cirrhosis patients with and without esophageal varices.

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Importance of the surrounding colonic mucosa in distinguishing between hyperplastic and adenomatous polyps during acetic acid chromoendoscopy

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Abstract

AIM: To examine the characteristics of colonic polyps, where it is difficult to distinguish adenomatous polyps from hyperplastic polyps, with the aid of acetic acid chromoendoscopy.

METHODS: Acetic acid spray was applied to colonic polyps smaller than 10 mm before complete excision. Endoscopic images were taken before and 15-30 s after the acetic acid spray. Both pre- and post-sprayed images were shown to 16 examiners, who were asked to interpret the lesions as either hyperplastic or adenomatous polyps. Regression analysis was performed to determine which factors were most likely related to diagnostic accuracy.

RESULTS: In 50 cases tested by the 16 examiners, the overall accuracy was 62.4% (499/800). Regression analysis demonstrated that surrounding colonic mucosa was the only factor that was significantly related to accuracy in discriminating adenomatous from hyperplastic polyps ($P < 0.001$). Accuracy was higher for polyps with linear surrounding colonic mucosa than for those with nodular surrounding colonic mucosa ($P < 0.001$), but was not related to the shape, location, or size of the polyp.

CONCLUSION: The accuracy of predicting histology is significantly related to the pattern of colonic mucosa surrounding the polyp. Making a histological diagnosis of colon polyps merely by acetic acid spray is helpful for colon polyps with linear, regularly patterned surrounding colonic mucosa, and less so for those with nodular, irregularly patterned surrounding colonic mucosa.

INTRODUCTION

The management of neoplastic and non-neoplastic colonic polyps is quite different^[1]. Therefore, it is of great interest for a colonoscopist to distinguish them during colonoscopic examination without having to take a biopsy sample. In addition, it takes a few days to establish a histological diagnosis from a biopsy sample, and there is no assurance that the pathological result of the biopsied specimen represents the lesion as a whole^[2]. The accuracy of conventional colonoscopy in distinguishing neoplastic from non-neoplastic lesions is reported to be between 68% to 84% even in the hands of experienced colonoscopists^[3,4]. Therefore, several methods are usually required to make the distinction between hyperplastic and adenomatous colonic polyps colonoscopically, such as special techniques like high-resolution chromoendoscopy, magnifying colonoscopy, or narrow band imaging magnification^[5-9]. However, these techniques are not used routinely in clinical practice for various reasons, for example, (1) lack of the appropriate equipment, (2) time restrictions, and (3) additional cost not covered by the insurance company.

Acetic acid enables a detailed examination of colonic neoplasms during colonoscopic examination by breaking the disulfide bonds of mucus, thus revealing the detail of the mucosal surface and allowing an analysis of the pit

pattern of the colonic polyps^[10]. Acetic acid is a cheap, efficient, safe and convenient tool^[11,12]. Therefore, once its accuracy in distinguishing between neoplastic and non-neoplastic polyps is established, its use might allow a one-stage colon polypectomy. Unfortunately, there are only few published data on the accuracy of acetic acid chromoendoscopy in the diagnosis of colonic polyps, and most of the previous studies were performed in conjunction with other methods such as magnifying endoscopy or with indigo carmine spraying^[12].

It would be helpful to colonoscopists if the factors affecting the accuracy in distinguishing between adenomatous and hyperplastic colon polyps were established. In the present study we examined the characteristics of colonic polyps in relation to the surrounding mucosa with the aid of acetic acid chromoendoscopy.

MATERIALS AND METHODS

Patients

From April to June 2006, 35 patients, in whom colonic polyps were revealed out of 299 routine colonoscopic examinations, were included in the study. Only 35 patients were included in the study since 258 examinations revealed no polyp and 6 revealed polyps only greater than 1 cm. Polyps greater than 1 cm were not included due to the higher probability of associated malignancy requiring removal regardless of the acetic acid chromoendoscopic finding. Colonoscopy was performed by one of the two colonoscopists (JH Kim and SY Lee) at the Digestive Disease Center of Konkuk University Hospital (the colonoscope Olympus CF260, Olympus Corp., Tokyo, Japan, was used). All patients provided written informed consent prior to colonoscopy. None of the 35 patients refused acetic acid chromoendoscopy. This prospective study was approved by the institutional review board of Konkuk University School of Medicine, which agreed that the study was in accordance with the ethical guidelines of the Helsinki Declaration.

Acetic acid chromoendoscopy

When a colon polyp smaller than 1 cm was found, 5-10 mL of 1.5% acetic acid was sprayed onto the lesion from a side channel of the colonoscope. On full air inflation, several endoscopic images were taken before and 15-30 s after spraying. Once the images were taken, the polyps were completely resected either by polypectomy or by cold biopsy sampling. The resected specimens were examined by pathologists, who were unaware of the endoscopic findings.

Size and location of the polyps were recorded. With respect to shape, the polyps were classified as either sessile or non-sessile. After being sprayed with acetic acid, the patterns of colonic mucosa surrounding the polyps were classified as having either (1) a linear and regular pattern (Figure 1) or (2) a nodular and irregular pattern (Figure 2).

Selection of cases for the blind test (primary analysis)

After the final pathology report, colon polyps other than adenomatous or hyperplastic polyps were excluded

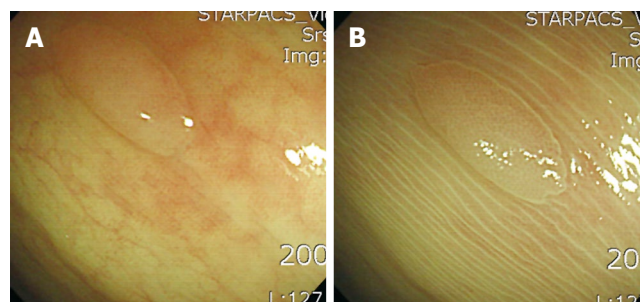


Figure 1 Linear and regularly patterned colonic mucosa surrounding a polyp. On endoscopic removal, pathology revealed a hyperplastic colon polyp. **A:** Before spraying with acetic acid a sessile polyp is seen; **B:** After spraying with acetic acid the colonic mucosa surrounding the polyp has a linear and regular pattern.

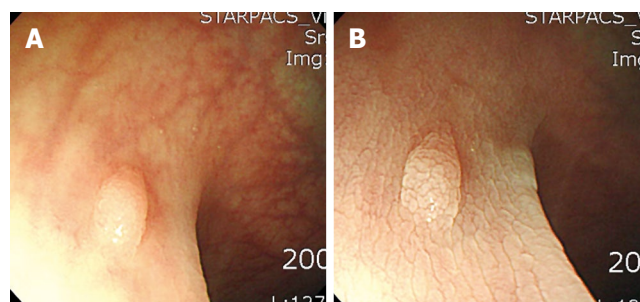


Figure 2 Nodular and irregularly patterned colonic mucosa surrounding a polyp. On endoscopic removal, pathology revealed a hyperplastic colon polyp. **A:** Before spraying with acetic acid a sessile polyp is seen; **B:** After spraying with acetic acid the colonic mucosa surrounding the polyp has a nodular and irregular pattern.

from the study. In our 35 patients, a total number of 54 colonic polyps smaller than 10 mm were detected during the colonoscopic examination. However, four cases were excluded since three were inflammatory polyps and one was a leiomyoma. The endoscopic images were selected by the colonoscopist who was not scheduled for the blind test (Lee SY). Finally, endoscopic images of 50 polyps, both pre- and post-sprayed, were collected.

Blind test by 16 examiners (secondary analysis)

A total of 100 endoscopic images, pre- and post-acetic acid sprayed images of each of the 50 colonic polyps, were shown to 16 examiners (6 gastroenterologists who were not familiar with the colonic pit patterns, 5 residents, and 5 medical students) at the same time in the same place. Before the blind test, a short, 10-min lecture on colonic pit patterns, that included presentation of 24 PowerPoint slides, was given by the colonoscopist who had selected the images for examination (SY Lee). Typical images of mucosal pit patterns in hyperplastic colon polyps (star-like or papillary-like pattern) and adenomatous colon polyps (tubular or gyrus-like pattern) were shown during the lecture^[13]. In addition, 10 cases of acetic acid sprayed images of colon polyps were shown to the 16 examiners. None of these images were included in the blind test and all of them were taken in Konkuk University Hospital only colonoscopically without using any other method.

The blind test was performed by showing the PowerPoint slides to the examiners. After examining two images

Table 1 Relationship between the colon polyp and the pattern of colonic mucosa surrounding the polyp *n* (%)

	Linear and regular (<i>n</i> = 34)	Nodular and irregular (<i>n</i> = 16)	<i>P</i> value
Size of the polyp (mm, mean ± SD)	5.29 ± 2.18	4.75 ± 2.27	NS
Shape of the polyp			0.023
Sessile	20 (58.8)	15 (93.7)	
Non-sessile	14 (41.2)	1 (6.3)	
Location of the polyp			NS
Cecum	2 (5.9)	0 (0.0)	
Ascending colon	5 (14.7)	2 (12.4)	
Hepatic flexure	3 (8.8)	0 (0.0)	
Transverse colon	1 (2.9)	1 (6.3)	
Splenic flexure	0 (0.0)	1 (6.3)	
Descending colon	4 (11.8)	1 (6.3)	
Sigmoid colon	12 (35.3)	8 (50.0)	
Rectum	7 (20.6)	3 (18.7)	
Pathology			NS
Hyperplastic polyp	10 (29.4)	7 (43.8)	
Adenomatous polyp	24 (70.6)	9 (56.2)	

NS: Not significant.

(one pre-acetic acid sprayed image and one post-acetic acid sprayed image) of each of the cases, the examiners were required to record their interpretation as either hyperplastic or adenomatous polyps. After the blind test, the responses were collected, transcribed, and analyzed for raw data associations.

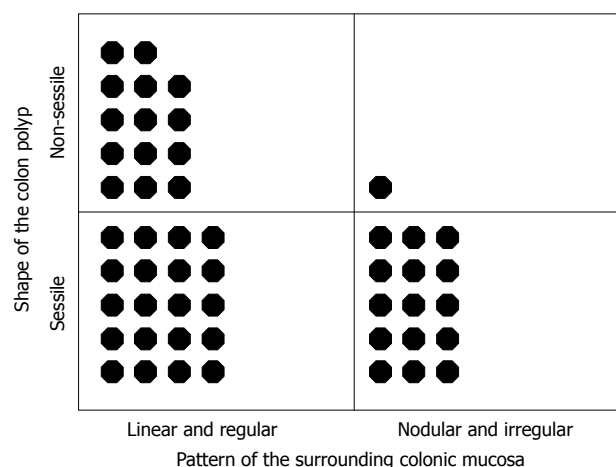
Statistical analysis

A *P*-value of less than 0.05 was considered statistically significant. Differences between the groups were analyzed using the chi-square tests and Student's *t*-tests. Regarding age and polyp size, results were expressed as mean ± SD. Regression analysis was performed to assess the accuracy of predicting pathology (adenomatous polyp versus hyperplastic polyp). Binary logistic regression analysis was performed with the factors which colonoscopists can notice during the colonoscopic examination, i.e., (1) shape of the polyp, (2) size of the polyp, (3) location of the polyp, and (4) surrounding colonic mucosa.

RESULTS

In the 50 cases tested by 16 examiners, the overall accuracy was 62.4% (499/800). There was no significant difference in test scores between the gastroenterologist group (30.3 ± 2.0, mean ± SD), the resident group (32.4 ± 5.4), and the medical student group (31.0 ± 5.6). Sensitivity, specificity, positive predictive value, and negative predictive value of acetic acid spray for adenomatous polyp were 81.8%, 41.2%, 73.0%, and 53.8%, respectively.

By regression analysis, the pattern of the surrounding colonic mucosa (*P* < 0.001) was the only factor predicting pathology (i.e. adenomatous polyp versus hyperplastic polyp). In 34 cases (68%), the colonic mucosa surrounding the polyp was linear and regular (Figure 1), and in 16 cases (32%) nodular and irregular (Figure 2). In contrast, the accuracy of distinguishing between hyperplastic and

**Figure 3** Diagnosis according to the shape of the colon polyp and the pattern of its surrounding colonic mucosa. Each black dot indicates a polyp. Most of the non-sessile colon polyps revealed linear and regular patterned surrounding colonic mucosa (*P* = 0.02).

adenomatous colon polyps was not related to the shape, location, or size of the polyp.

Neither age nor sex of the patient was related to the pattern of colonic mucosa surrounding the polyp (Table 1). The only related factor was the shape of the polyp (*P* = 0.02; Figure 3). Whereas size and location of the polyp were irrelevant.

DISCUSSION

To the best of our knowledge, this study is the first to evaluate the significance of the type of colonic mucosa surrounding a colon polyp for distinguishing between adenomatous and hyperplastic polyps during acetic acid chromoendoscopy. Our findings revealed that linear and regularly patterned surrounding colonic mucosa enables a higher accuracy in predicting the pathology of the associated polyp when compared to those surrounded by nodular and irregularly patterned colonic mucosa.

Although the reasons for these two different surrounding colonic mucosal patterns are unclear, the nodular pattern seems to be similar to that seen in acetic acid sprayed gastric mucosa, which is considered to be indicative of chronic mucosal damage induced either by *H. pylori* infection or by acid irritation (unpublished data). In the present study, the pattern of the mucosa surrounding a polyp was associated with the shape of the polyp, the non-sessile type being found more frequently in linear patterned surrounding colonic mucosa. However, this should be evaluated further by a large-scale study in conjunction with pathological analysis.

Most of the previous studies on acetic acid chromoendoscopy have examined the significance of detecting sessile polyps or analyzed polyp pit patterns^[5,10-12]. In the present study, we did not classify colonic adenomas according to their degree of dysplasia, but simply assessed the accuracy of acetic acid chromoendoscopy, which is a cheap, easy, convenient, safe and fast procedure, in distinguishing between adenomatous and hyperplastic

colon polyps. We therefore analyzed only the mucosal pit patterns, and not the microvascular pattern, which is automatically masked by spraying with acetic acid.

It has been reported that residents are able to safely and effectively screen for colorectal neoplasms with a flexible sigmoidoscope when supervised^[14]. Interestingly, there was no significant difference in the blind test scores between the gastroenterologists, residents, and medical students. This indicates that the results achieved using acetic acid chromoendoscopy are easy to interpret, even for those who have no experience in gastrointestinal endoscopy. However, it also indicates that although uniform descriptions of colonic mucosal pit patterns in hyperplastic colon polyps (star-like or papillary-like pattern) and in adenomatous colon polyps (tubular or gyrus-like pattern) are useful, they are not completely visualized merely by acetic acid chromoendoscopy.

The limitation of our study is that this was the data from 16 examiners, who were not familiar with the colonic pit patterns, predicted the pathology only on the basis of 2 pictures (pre- and post-sprayed images) not by full colonoscopic examination. Moreover, no additional methods such as magnifying endoscopy or indigo carmine spray were used. Therefore, the overall diagnostic accuracy for distinguishing between neoplastic and non-neoplastic lesions was lower than previous studies which were done by experienced colonoscopists^[3,9,15].

In conclusion, acetic acid chromoendoscopy can be used to distinguish between hyperplastic and adenomatous polyps without magnifying endoscopy with an accuracy of 62.4%, and the accuracy is significantly related to the pattern of colonic mucosa surrounding the polyp. In addition, we have found that making a histological diagnosis of colon polyps merely by acetic acid spray is helpful for colon polyps with linear, regularly patterned surrounding colonic mucosa, and less so for those with nodular, irregularly patterned surrounding colonic mucosa.

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COMMENTS

Background

The management of neoplastic and non-neoplastic colonic polyps is quite different, and it is of great interest for a colonoscopist to distinguish them during colonoscopic examination without having to take a biopsy sample. Acetic acid is a cheap, efficient, safe and convenient tool which enables a detailed examination of colonic neoplasms during colonoscopic examination by breaking the disulfide bonds of mucus. Acetic acid chromoendoscopy is effective in revealing the detail of the mucosal surface and allowing an analysis of the pit pattern of the colonic polyps.

Research frontiers

Recently, several endoscopic methods that help to make the distinction between hyperplastic and adenomatous colonic polyps colonoscopically have been introduced. Special techniques such as high-resolution chromoendoscopy, magnifying colonoscopy, or narrow band imaging magnification are being innovated.

Innovations and breakthroughs

This is the first study that evaluated the significance of the type of colonic mucosa surrounding a colon polyp for distinguishing between adenomatous and hyperplastic polyps during acetic acid chromoendoscopy. Our findings revealed that linear and regularly patterned surrounding colonic mucosa enables a higher accuracy in predicting the pathology of the associated polyp when compared to those surrounded by nodular and irregularly patterned colonic mucosa.

Applications

Through this study, we showed a new way to distinguish between neoplastic and non-neoplastic polyps by acetic acid chromoendoscopy which is a cheap, easy, convenient, safe and fast procedure. The patterns of surrounding colonic mucosa will help colonoscopists in distinguishing between adenomatous and hyperplastic colon polyps.

Terminology

Acetic acid chromoendoscopy is a method done by spraying 5-10 mL of 1.5% acetic acid onto the lesion from a side channel of the colonoscope. Endoscopic images are usually taken before and 15-30 s after spraying.

Peer review

This study interpreted the lesions as either hyperplastic or adenomatous polyps after acetic acid chromoendoscopy, and proved that making a histological diagnosis of colon polyps merely by acetic acid spray is helpful for colon polyps with linear, regularly patterned surrounding colonic mucosa, and less so for those with nodular, irregularly patterned surrounding colonic mucosa.

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RAPID COMMUNICATION

Hemodynamic effects of propranolol with spironolactone in patients with variceal bleeds: A randomized controlled trial

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Abstract

AIM: To study the hemodynamic effects of spironolactone with propranolol *vs* propranolol alone in the secondary prophylaxis of variceal bleeding.

METHODS: Thirty-five cirrhotics with variceal bleeding randomly received propranolol ($n = 17$: Group A) or spironolactone plus propranolol ($n = 18$: Group B). Hemodynamic assessment was performed at baseline and on the eighth day.

RESULTS: Spironolactone with propranolol caused a greater reduction in the hepatic venous pressure gradient than propranolol alone (26.94% *vs* 10.2%; $P < 0.01$). Fourteen out of eighteen patients on the combination treatment had a reduction in hepatic venous pressure gradient to ≤ 12 mmHg or a 20% reduction from baseline in contrast to only six out of seventeen (6/17) on propranolol alone ($P < 0.05$).

CONCLUSION: Spironolactone with propranolol results in a better response with a greater reduction in hepatic venous pressure gradient in the secondary prophylaxis of variceal bleeding. A greater number of patients may be protected by this combination therapy than by propranolol alone. Hence, this combination may be recommended for secondary prophylaxis in patients with variceal bleeding.

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Key words: Hepatic venous pressure gradient; Secondary

prophylaxis; Spironolactone; Propranolol; Variceal bleeding

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De BK, Dutta D, Som R, Biswas PK, Pal SK, Biswas A. Hemodynamic effects of propranolol with spironolactone in patients with variceal bleeds: A randomized controlled trial. *World J Gastroenterol* 2008; 14(12): 1908-1913 Available from: URL: <http://www.wjgnet.com/1007-9327/14/1908.asp> DOI: <http://dx.doi.org/10.3748/wjg.14.1908>

INTRODUCTION

Variceal hemorrhage is one of the serious complications of portal hypertension in cirrhotics. About 70% of the survivors of variceal bleeds re-bleed within a year^[1,2]. Of the various treatment modalities, pharmacotherapy is one of the more attractive avenues, as it is simple and safe and can be applied outside the hospital.

Beta-blockers like propranolol are the treatment of choice for primary prophylaxis of variceal hemorrhage^[3]. Reduction of the hepatic venous pressure gradient (HVPG) to ≤ 12 mmHg is protective, while reduction by greater than or equal to 20% of the baseline value is also safe^[4,5].

However, only about one-third of patients taking propranolol alone achieve such reductions among bleeders^[4,6]. Thus, a considerable number of patients are not protected by propranolol alone, particularly for secondary prophylaxis.

Hence drug combinations have been advocated for prevention of variceal re-bleeding. Recently, a combination of isosorbide mononitrate with propranolol has been tested for the prevention of variceal re-bleeding, with some benefit^[7]. There have been a few studies showing efficacy of spironolactone (a drug that is commonly prescribed in cirrhotics with ascites) in the reduction of portal pressure among cirrhotics without ascites^[8-11]. We evaluated the hemodynamic effects of a combination therapy of propranolol with spironolactone and found the combination had greater efficacy than propranolol alone, in propranolol-resistant cases^[12]. Considering these facts, we have examined the portal hemodynamic effects of propranolol alone and propranolol in combination with spironolactone among cirrhotics who have bled at least once.

MATERIALS AND METHODS

Forty-two consecutive liver cirrhosis patients with variceal bleeding were enrolled from the Liver Clinic of Medical College and Hospital Calcutta. This study was carried out from June 2005 to March 2007. Hemodynamic studies were undertaken in the catheter laboratory of The Institute of Cardiovascular Sciences, RG Kar Medical College and Hospital, Calcutta, which is situated close to Medical College Calcutta. The institutional ethics committees of both the hospitals approved the study protocol. Only those patients who had experienced at least one episode of variceal bleeding within the previous week were considered. After admission to hospital due to upper gastrointestinal bleeding with clinical features of chronic liver disease, patients underwent upper gastrointestinal endoscopy on the following day. Those patients who came with acute bleeding were initially treated with vasoconstrictors (like terlipressin), plasma expanders or blood transfusions, as and when necessary, and endoscopy was performed twenty-four hours after their hemodynamic stabilization. In the event of re-bleeding, endoscopic banding was performed. Only those patients with evidence of active variceal bleeding or clots or oozing from the varices were included. In addition, those patients with upper gastrointestinal bleeding who had esophageal varices in the absence of any other source of upper gastrointestinal bleed were also included. Patients were excluded for any of the following reasons: Asthma, congestive cardiac failure, severe diabetes, severe hypertension, any severe co-morbid states, age less than 15 years or more than 70 years, or previous treatment with endoscopic sclerotherapy, variceal ligation, porto-systemic shunt surgeries, beta-adrenergic blocking agents, spironolactone or nitrates. The procedures to be employed were explained to the patients and, after obtaining informed written consent, patients remained hospitalized for the duration of the study. All patients underwent blood tests to evaluate the liver chemistry (liver function tests, prothrombin time) and to establish the etiology of chronic liver disease (viral markers, anti-nuclear factor, ceruloplasmin). They also underwent routine investigations (complete hemogram, urea, creatinine, random sugar, electrolytes), ultrasonography with Doppler study and liver biopsy (as and when necessary) to establish the diagnosis. Variceal grading was adopted as per the Japanese Research Society^[13]. Thereafter, the patients were transferred to The Institute of Cardiovascular Sciences, RG Kar Medical College for hemodynamic assessment. The first hemodynamic study was performed within a week of the last variceal bleeding episode.

Hemodynamic study

All patients received a normal hospital diet, without any sodium restriction. Hemodynamic studies were carried out in all cases within a week of variceal bleeding after an overnight fast using a standard technique^[14] in the catheter laboratory of the Institute of Cardiovascular Sciences RG Kar Medical College, Calcutta. Under local anesthesia in the supine position, a venous introducer was placed in the right femoral vein by the Seldinger technique. Under fluoroscopic guidance (Axiom Artis; Siemens, Munich Germany), a 7F balloon-tipped catheter (USCI; CR Bard Ire-

land, Galway, Ireland) was introduced into the main right hepatic vein through the inferior vena cava (IVC). Free (FHVP) and wedged (occluded) (WHVP) hepatic venous pressures were measured using a hemodynamic monitor (Axiom Sensis Germany) with pressure transducers (SIEMENS HEMOMED). Thereafter, we also measured the pressures in the IVC, right atrium (RA), mean pulmonary arterial pressure (MPAP) and pulmonary capillary wedge pressure (PCWP) in the similar fashion, wherever possible. Hepatic venous pressure gradient (HVPG) was calculated as the difference between WHVP and FHVP. All measurements were made in triplicate and means were obtained (data were recorded from the tracer curves).

After the baseline readings, the patients were divided into two groups by a computer-generated randomized table. One group (Group A) received propranolol (Inderal; ICI Pharmaceuticals, Chennai) at a dose of 40 mg twice daily and a placebo tablet in place of spironolactone, and the other group (Group B) received propranolol (40 mg twice daily) with spironolactone (Aldactone; 100 mg once daily; Searle: India, Mumbai). The dose of propranolol was gradually increased until a twenty percent reduction from the baseline pulse rate, or a pulse rate of 60 was achieved, whichever came earlier. Hemodynamic studies were repeated on the eighth day, after the morning dose. The patients, the investigators, the cardiologist and the statistician conducting the hemodynamic study were blinded to the nature of the treatment given.

Responders were defined as those individuals showing a reduction of HVPG to ≤ 12 mmHg and/or greater than a 20% reduction in HVPG from the baseline (primary outcome)^[5].

Forty-two patients with clinical features of chronic liver disease were initially considered and had upper gastrointestinal endoscopy. Of these, thirty-eight patients were considered for hemodynamic assessment. Of the four patients excluded, two had chronic obstructive lung disease, one had uncontrolled diabetes mellitus and the last was a case associated with peptic ulcer disease. Of these thirty-eight patients, thirty-five patients were included in the final analysis. Three more patients were excluded, as one of them had severe re-bleeding for which emergency endoscopic therapy was performed before hemodynamic assessment could be undertaken and the other two declined the second reading.

Statistical analysis

Results are expressed as mean \pm SD. Chi-square tests, correlation and regression, paired *t* test and single factor ANOVA were used as required. *P* < 0.05 was considered statistically significant.

RESULTS

Thirty-five cirrhotics with variceal bleeding were included in the final analysis. The clinical and biochemical profiles of the included patients are given in Table 1. No subject had varices less than grade II. Sixteen of our cases (45.71%) were alcoholics; nine (25.71%) were of viral etiology, in eight patients (22.86%) no etiology was found, and there was one each of Wilson's and autoimmune liver disease.

Table 1 Clinical and biochemical profiles of patients on propranolol or propranolol and spironolactone

Characteristics	Group A <i>n</i> = 17	Group B <i>n</i> = 18	<i>P</i> value
Male:Female ratio	12:5	15:3	
Age (yr)	44.3 ± 7.98	46.61 ± 8.71	0.09
Ascites	10	8	
Encephalopathy	1	2	
Etiology			
Alcoholic	6	10	
Hepatitis-B	6	2	
Hepatitis-C	0	1	
Others	5	5	
Varices			
II	3	4	
III	11	9	
IV	3	5	
Child's score			
A	3	4	
B	8	9	
C	6	5	
Bilirubin (mg/dL)	2.26 ± 1.8	2.13 ± 2.13	0.85
Albumin (mg/dL)	2.89 ± 0.46	2.71 ± 0.78	0.42
Globulin (mg/dL)	3.72 ± 0.76	4.64 ± 2.26	0.12
ALT (IU/L)	79.35 ± 75.43	43.89 ± 18.20	0.08
Urea (mg/dL)	29.53 ± 11.03	28.6 ± 13.57	0.83
Creatinine (mg/dL)	0.88 ± 0.27	0.84 ± 0.28	0.67
Na ⁺ (mEq/L)	135.7 ± 4.9	133.22 ± 4.61	0.13
K ⁺ (mEq/L)	3.74 ± 0.56	3.66 ± 0.49	0.64
Prothrombin time (INR)	1.21 ± 0.16	1.34 ± 0.40	0.30
Dose of propranolol (mg/d)	92.94 ± 23.39	88.89 ± 20.83	0.59

Values are shown as mean ± SE, *P* < 0.05 considered statistically significant. Group A: Propranolol; Group B: Propranolol and Spironolactone; ALT: Alanine aminotransferase.

There was no statistically significant difference in the clinical and biochemical profiles between Group A (only propranolol) and Group B (propranolol with spironolactone), as shown in Table 1.

In Group A, there was a significant reduction in HVPG after therapy, as compared with the baseline (*P* < 0.01, Table 2). The differences in other hemodynamic parameters before and after propranolol administration were not statistically significant. Interestingly, in Group A, there was a paradoxical rise in HVPG in 5 patients (45.45%) among the non-responders (11 patients). We also observed a rise in FHVP in 10 out of the 17 patients on propranolol.

In Group B, there were significant reductions in both WHVP and HVPG after therapy compared with the baseline (*P* < 0.001, Table 2). None of the patients showed an increase in HVPG after drug therapy in contrast to five patients in Group A. We also observed an increase in FHVP among 9 of the 18 patients on propranolol with spironolactone.

Comparing Group A with Group B, 6 of the 17 patients (35.29%) in Group A and 14 of the 18 patients (77.78%) in Group B showed an HVPG reduction to either ≤ 12 mmHg or at least a 20% reduction from the baseline (responder) (*P* = 0.011). Interestingly, 6 of the 17 patients (35.29%) in Group A and 13 of the 18 patients (72.2%) in Group B showed a 20% reduction in HVPG from the baseline (*P* = 0.0283). Among these, 5 patients

(29.41%) in Group A and 11 patients (61.11%) in Group B had an absolute reduction in HVPG to ≤ 12 mmHg (*P* = 0.0599).

The percent reductions in HVPG from baseline after a seven-day therapy were 10.2% and 26.94% in Group A and Group B, respectively, which was also statistically significant (*P* < 0.05, Table 2).

Compared with the baseline, post-drug right atrial pressures increased significantly among the responders (5.2 mmHg *vs* 6.1 mmHg, *P* < 0.05) in contrast to the non-responders (4.73 mmHg *vs* 5.87 mmHg, *P* = 0.12).

Interestingly, analyzing the baseline hemodynamic parameters in responders, we observed a strong inverse correlation between HVPG and MPAP (*r* = -0.58) and a moderate inverse correlation between PCWP (*r* = -0.48) and RA pressures (*r* = -0.30). However after drug treatment, these relationships ceased to exist. By contrast, among non-responders, no correlation was observed between the baseline HVPG and any of MPAP (*r* = -0.141), PCWP (*r* = -0.069) and RA pressures (*r* = -0.0007).

DISCUSSION

For pharmaco-prophylaxis of variceal bleeding, most drugs act by vasoconstriction to reduce portal pressure. Increased blood volume, a common feature in decompensate cirrhosis, has a contributory effect in increasing portal pressure. It has been found acute expansion of blood volume by transfusion increases the chances of re-bleeding in a bleeder^[15]. Moreover, increased blood volume maintains the hyperdynamic state of portal hypertension^[16]. Thus, a reduction in plasma volume may reduce portal pressure. Spironolactone, a mineralocorticoid-blocking agent is used for its ability to reduce portal pressure as measured by HVPG^[8-10]. Thus, the combination of spironolactone with a beta-blocker may reduce the portal pressure in a better way. The rationality behind the use of combination therapy is that effects acting through different mechanisms may be additive or even synergistic^[17].

In this study, a significantly larger number of patients responded to combination therapy than responded to propranolol alone (14/18 *vs* 6/17, *P* < 0.05).

A better response with spironolactone was observed in a subset of eight non-ascitic patients who did not respond to propranolol for primary prophylaxis^[11]. Variceal pressure was measured by endoscopy in that study. We also observed in our previous study that spironolactone when combined with propranolol reduced portal pressure in propranolol-resistant cases (measuring HVPG)^[12].

Studies have shown spironolactone does not further reduce portal pressure in patients already on low-dose transdermal nitroglycerine^[18] or beta-blockers like nadolol^[19] for primary prophylaxis.

The most important observation in our study is the significantly larger number of patients on combination therapy showing either an absolute reduction in HVPG to ≤ 12 mmHg or at least a 20% reduction from the baseline (responder) compared with those on propranolol alone (*P* = 0.011).

A landmark paper by Feu *et al*^[5] observed for the first time that a reduction in HVPG of more than 20% of

Table 2 Hemodynamic parameters at baseline and on d 8 of therapy

Characteristics	Group A (n = 17)			Group B (n = 18)			Group A vs Group B P-value	
	Baseline	d 8	P-value	Baseline	d 8	P-value	Baseline	d 8
Pulse rate (/min)	86.80 ± 12.0	69.17 ± 8.66	0.058	79.89 ± 10.21	64.66 ± 8	0.00003	0.08	0.12
SBP (mmHg)	123.88 ± 11.82	117.41 ± 9.45	0.0003	123.76 ± 11.3	118.44 ± 8.85	0.001	0.98	0.74
IVCP (mmHg)	7.29 ± 3.33	7.19 ± 2.95	0.89	7.33 ± 3.6	7.11 ± 3.34	0.74	0.97	0.95
FHVP (mmHg)	7.88 ± 3.12	8.82 ± 3.99	0.28	8.22 ± 3.64	9.00 ± 3.14	0.25	0.76	0.86
WHVP (mmHg)	24.65 ± 3.23	23.88 ± 5.11	0.54	24.56 ± 4.3	20.78 ± 3.83	0.00009	0.94	0.052
HVPG (mmHg)	16.76 ± 2.66	15.06 ± 4.35	0.04	16.11 ± 1.97	11.78 ± 2.07	-	-	-
RAP (mmHg)	4.88 ± 2.87	6.25 ± 2.57	0.007	5.11 ± 2.59	5.78 ± 2.39	0.27	0.81	0.58
MPAP (mmHg)	17.46 ± 3.91	19.58 ± 4.8	0.32	18.11 ± 4.43	18.80 ± 4.29	0.60	0.76	0.82
PCWP (mmHg)	14.54 ± 6.78	14.33 ± 6.82	0.94	15.50 ± 4.53	14.40 ± 4.95	0.48	0.69	0.98

All the values are shown as mean ± SE. $P < 0.05$ considered statistically significant. SBP: Systolic blood pressure; IVCP: Inferior vena cava pressure; FHVP: Free hepatic venous pressure; WHVP: Wedge hepatic venous pressure; HVPG: Hepatic venous pressure gradient; RAP: Right atrial pressure; MPAP: Mean pulmonary artery pressure; PCWP: Pulmonary capillary wedge pressure; Group A: Propranolol; Group B: Propranolol and Spironolactone.

baseline, even if not reaching the 12 mmHg target, is associated with almost complete protection against variceal re-bleeding. Eight studies^[5,20-26], either RCTs or prospective consecutive series, have shown the pharmacologic (or spontaneous) reduction of HVPG to less than 12 mmHg, or by as much as or more than 20% of the baseline value, virtually abolishes the risk of re-bleeding.

As mentioned earlier, one study demonstrated that addition of isosorbide mononitrate improved the efficacy of propranolol in the prevention of variceal re-bleeding on long-term follow up^[7].

The combination of spironolactone with propranolol showed a significantly greater percent reduction of HVPG from the baseline, as compared with propranolol alone (26.94% *vs* 10.2, $P < 0.05$). This greater reduction in HVPG with combination therapy may in part be explained by a paradoxical rise in FHVP with a concomitant significant reduction in WHVP, in contrast to only a rise in FHVP without a significant post treatment reduction in WHVP in patients on propranolol alone. However, propranolol alone also significantly reduced HVPG from the baseline after 7-d therapy ($P < 0.05$).

Since ascites does not alter HVPG or the gradient between portal venous pressure and intra-abdominal pressure^[27,28], reduction of HVPG by the addition of spironolactone is likely to be due to a true reduction in portal venous pressure and not due to a reduction in intra-abdominal pressure consequent to control of the ascites. Moreover, spironolactone, by reducing plasma volume, may reduce both WHVP and FHVP; thus, it should not influence HVPG. The efficacy of spironolactone in the reduction of portal pressure in patients without ascites has already been demonstrated^[17]. Reduction of plasma volume and associated vasoactive mechanism may underlie the effects of spironolactone on portal pressure^[11]. However, some evidence suggests spironolactone may have a direct effect on the vasculature, independent of its anti-aldosterone effect^[29]. Spironolactone also has a unique property of inhibition of hepatic stellate cell activation and Na/H exchange isoform-1 (NHE-1) protein expression^[30]. Spironolactone was shown to have a mineralocorticoid receptor-independent suppressive effect on immuno-active and inflammatory cytokines^[31]. An anti fibrotic property

has also been evidenced experimentally in rats^[30]. Recent studies have also demonstrated the aldosterone antagonist eplerenone prevents epithelial cell growth and stiffening of venous and arterial endothelia^[32].

Although most studies evaluated the effects of spironolactone over a longer period of time (4-8 wk), we completed our second hemodynamic reading after a week, considering that chance of re-bleeding after the index bleeding is maximal during the first two weeks. Moreover, one of the major active metabolites of spironolactone is canrenone, which has a slow clearance and a half-life of 10-35 h. Thus, to reach a steady state plasma concentration it would take a period of between 2-7 d.

Incidentally we observed a significant rise in right atrial pressures only among the responders of both groups following drug therapy. Moreover, there was a moderate to strong inverse correlation between the baseline HVPG and the baseline MPAP, PCWP and RA pressures, only among the responders. The significance or role of this observation needs further evaluation.

Hence, spironolactone, a drug commonly prescribed in cirrhotics for the reduction of ascites, has a potential independent portal pressure-reducing effect, and its impressive reduction of HVPG in combination with propranolol may pave our way to recommend this combination for secondary prophylaxis in variceal bleeding.

COMMENTS

Background

Variceal bleeding is one of the potentially life threatening complications of portal hypertension. About 70% of the survivors of variceal bleeds re-bleed within one year. Beta-blockers like propranolol have been the treatment of choice for prevention of variceal bleeding. However, only about one-third of the patients taking propranolol achieve a significant reduction in the hepatic venous pressure gradient to be considered risk free. Hence, drug combinations have been advocated for the prevention of variceal bleeding.

Research frontiers

Various drug combinations have been tested for the prevention of variceal bleeding; for example, propranolol with isosorbide mononitrate. However, the problem with drug combinations is an increased incidence of side effects, which leads to discontinuation of therapy. The challenge is to find a drug combination that is not only effective but also safe and easy to administer over long periods of time.

Innovations and breakthroughs

Spironolactone, a drug commonly used in cirrhotics with ascites to reduce fluid overload, has been found to have an independent portal hypotensive effect. The drug has been in use for a long period of time and has been found to be safe and free of side effects except for occasional gynaecomastia. The idea was to study the hemodynamic changes induced by the combination of spironolactone with propranolol, and compare it with propranolol alone, the gold standard drug. The significantly better response of patients receiving this combination pharmacotherapy (spironolactone plus propranolol) for secondary prophylaxis of variceal bleeding may be considered as a breakthrough.

Applications

We found the combination of spironolactone with propranolol resulted in a significantly greater reduction in HVP than propranolol alone, and this reduction was significant enough to cause patients to be relatively risk free from recurrence of variceal bleeds. However, long-term prospective studies are needed in a larger number of patients to actually observe the recurrence of variceal bleeding, if any.

Terminology

The hepatic venous pressure gradient (HVPG) is measured by the introduction of a balloon-tipped catheter into the hepatic vein. HVPG is a very strong marker of the degree of portal hypertension.

Peer review

This is a very interesting study dealing with a significant clinical problem. It is well conducted and most significant issues are addressed.

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RAPID COMMUNICATION

Effect of *H pylori* infection and its eradication on hyperammonemia and hepatic encephalopathy in cirrhotic patients

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Abstract

AIM: To investigate the relationship between *H pylori* infection, blood ammonia concentration and hepatic encephalopathy (HE), and the effect of *H pylori* eradication in cirrhotic patients.

METHODS: From July 2003 to January 2005, 457 cirrhotic patients in five regions of Zhejiang Province were enrolled. Patients were evaluated for demographics, number connection test, *H pylori* infection, liver impairment, blood ammonia concentration and HE. Patients with *H pylori* infection were given 1 wk therapy with omeprazole plus clarithromycin and tinidazole. ¹⁴C urea breath test was performed and mental symptoms and blood ammonia level were reassessed after bacterium eradication.

RESULTS: Overall *H pylori* infection rate was 60.6%, and HE occurred in 47.5% of cirrhotic patients. Subclinical HE (SHE) was detected in 55 of 117 cirrhotic patients. Blood ammonia concentration in *H pylori* negative ($n = 180$) and positive ($n = 277$) cirrhotic patients was 53.8 ± 51.4 and 78.4 ± 63.6 $\mu\text{mol/L}$, respectively ($P < 0.01$), which was significantly reduced to 53.5 ± 37.7 $\mu\text{mol/L}$ after bacterium eradication ($n = 126$) ($P < 0.01$). Blood ammonia was 97.5 ± 81.0 $\mu\text{mol/L}$ in *H pylori*-positive cirrhotic patients, and this did not significantly change in those with persistent infection after *H pylori* eradication ($n = 11$). HE was more frequently observed in patients with *H pylori* infection than in those without (58.5% vs 30.6%, $P < 0.01$). HE rate significantly dropped to 34.1% after *H pylori* eradication ($P < 0.01$). *H pylori* prevalence significantly differed among cirrhotic patients with HE (74.4%), SHE

(69.1%), and those without HE (53.2%) ($P < 0.05$). Blood ammonia level was significantly different among cirrhotic patients with HE (94.5 ± 75.6 $\mu\text{mol/L}$), SHE (59.9 ± 49.2 $\mu\text{mol/L}$), and without HE (47.3 ± 33.5 $\mu\text{mol/L}$) ($P < 0.05$). Logistic regression analysis showed that blood ammonia concentration, Child-Pugh stage, upper gastrointestinal bleeding, electrolyte disturbance, and urea nitrogen were risk factors for HE.

CONCLUSION: *H pylori* infection is an important factor for inducing high blood ammonia concentration and HE in cirrhotic patients. *H pylori* eradication may be helpful for treatment and prevention of HE.

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Key words: Cirrhosis; *Helicobacter Pylori*; Hepatic encephalopathy; Hyperammonemia

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INTRODUCTION

Hepatic encephalopathy (HE) is a frequent complication of liver cirrhosis. Although the pathogenesis is unclear, ammonia is one of the key factors involved. Recently, it has been suggested *H pylori* contributes to hyperammonemia in cirrhosis, and bacterium eradication decreases blood ammonia concentration in these patients^[1-8]. However, the literature contains conflicting data, with several other studies showing ammonia levels do not significantly differ between cirrhotic patients with and without *H pylori* infection. Ammonia production in the stomach by *H pylori* urease appears to be inadequate to clinically affect ammonia disposal in the majority of cirrhotic patients^[2,9-13]. The possible role of *H pylori* in the pathogenesis of HE deserves further investigation.

MATERIALS AND METHODS

Subjects

From July 2003 to January 2005, 457 cirrhotic patients in 18 hospitals from five regions of Zhejiang Province in China were enrolled in this prospective study. Diagnosis of liver cirrhosis was carried out by history, clinical examination, laboratory findings, and radiological findings according to the principles established by Chinese Hepatology Association in 2002. The main exclusion criteria included: (1) Severe cardiac, pulmonary, cerebral and renal disorders; (2) severe HE of grades III and IV; (3) currently receiving *H. pylori* eradication therapy; (4) currently undergoing surgery, (5) active gastrointestinal bleeding where non-surgical therapy is ineffective; (6) psychological disorders other than HE; and (7) current alcohol or sedative-drug abuse.

Patients were evaluated for demographic checklists, number connection test (NCT), *H. pylori* infection, liver impairment (according to Child-Pugh classification, including the total score of HE, ascites, prothrombin time, albumin concentration and bilirubin level, which ranked as Child-Pugh class A, B and C), blood ammonia concentration, and HE status. All patients received a low-salt, low-protein diet, and lactulose was given to all patients to induce two to four bowel movements a day. Protein intake was restricted to about 20-40 g daily. One hundred and thirty-seven patients with *H. pylori* infection were given 1 wk eradication therapy. Mental symptoms and blood ammonia levels were reassessed 1 mo after eradication therapy.

Detection of *H. pylori* infection

Gastric specimens were taken from the antrum when performing endoscopic biopsies, which were assessed by rapid urease test, histology (Giemsa staining) or *H. pylori* culture. The presence of *H. pylori* was detected by ¹⁴C urea breath test in those who did not undergo biopsy. Subjects who had *H. pylori* were identified by at least one of the above tests showing a positive result.

Ammonia measurement

Fasting venous blood samples were obtained from each patient to measure ammonia concentration ($\mu\text{mol/L}$), according to the manufacturer's instructions.

NCT

The NCT (part A) was performed to detect subclinical HE. Subjects were required to connect numbers printed on paper consecutively from 1 to 25, as quickly as possible. NCT abnormality was defined as taking > 66 s to fulfill this task.

HE stage

HE stage was established by clinical characteristics, electroencephalography (EEG) and NCT results. Patients were classified as cirrhotic without HE, with subclinical HE (SHE), and with HE. SHE was characterized by normal traditional clinical evaluation with definite and quantifiable neuropsychological defects (NCT abnormality).

Table 1 Clinical characteristics of *H. pylori*-positive and -negative patients

	<i>H. pylori</i> (+)	<i>H. pylori</i> (-)	<i>P</i> value
Sex (male/female)	196/81	141/39	0.045
Age (yr)	57.6 \pm 12.7	56.9 \pm 13.4	0.604
Child-Pugh class			
A/B/C	67/124/86	55/77/48	0.309
Upper gastrointestinal hemorrhage	155	95	0.263
Hepatorenal syndrome	19	11	0.448
Ascites	197	136	0.208

H. pylori eradication therapy

The 137 cirrhotic patients with *H. pylori* infection received 7 d dual eradication therapy (omeprazole 20 mg b.i.d plus clarithromycin 500 mg b.i.d plus tinidazole 500 mg b.i.d). One month after completion of treatment, a ¹⁴C-urea breath test was performed to reassess *H. pylori* status.

Statistical analysis

Statistics were calculated using SPSS ver. 11.0. Qualitative variables were expressed by means of frequency and percentiles, and were analyzed using the χ^2 test. Quantitative results are expressed as means \pm SD. Groups were compared by using Student's *t* test or ANOVA. Risk factors for HE were analyzed using logistic multiple regression. Odds ratio (OR) values were calculated from 95% CI, and OR > 1.00 was considered a significant risk factor. Statistical significance was established at *P* < 0.05.

RESULTS

Effect of *H. pylori* infection on blood ammonia and HE

Overall *H. pylori* infection rate was 60.6% (277/457). There were 137 *H. pylori*-positive patients who received eradication therapy, among whom the eradication rate was 91.4% (126/137). HE occurred in 47.5% of cirrhotic patients (217/457), and SHE was detected in 47.0% (55/117) of those without HE. There was no significant difference in liver impairment (Child-Pugh class), and complications (upper gastrointestinal bleeding, hepatorenal syndrome, and ascites) between *H. pylori* positive and negative groups (Table 1). Blood ammonia concentration in *H. pylori* negative and positive cirrhotic patients was 53.8 \pm 51.4 and 78.4 \pm 63.6 $\mu\text{mol/L}$, respectively (*P* < 0.01). Blood ammonia was 78.4 \pm 63.6 $\mu\text{mol/L}$ in *H. pylori*-positive cirrhotic patients before treatment (*n* = 137), and 97.5 \pm 81.0 $\mu\text{mol/L}$ in those with persistent infection after treatment (*n* = 11). Blood ammonia was significantly reduced to 53.5 \pm 37.7 $\mu\text{mol/L}$ (*P* < 0.01) in the *H. pylori* eradication group (*n* = 126). HE was more frequently observed in patients with *H. pylori* infection than in those without (58.5% *vs* 30.6%, *P* < 0.01). HE rate significantly dropped to 34.1% after *H. pylori* eradication (*P* < 0.01). Data are shown in Table 2.

Relationship between HE and *H. pylori* infection and blood ammonia

H. pylori prevalence differed significantly between cirrhotic patients with HE (74.4%), those with SHE (69.1%), or

Table 2 Effect of *H pylori* infection on blood ammonia concentration and HE

<i>H pylori</i> infection	<i>n</i>	Ammonia concentration (μmol/L)	HE rate
<i>H pylori</i> (-)	180	53.8 ± 51.4 ^{bd}	55 (30.6%) ^{bd}
<i>H pylori</i> (+)	277	78.4 ± 63.6	162 (58.5%)
Eradicated	126	53.5 ± 37.7 ^{bd}	43 (34.1%) ^{bd}
Failed to eradicate	11	97.5 ± 81.0	6 (54.5%)

^b*P* < 0.01, *vs* failed to eradicate (+) group; ^d*P* < 0.01, *vs H pylori* (+) group.

Table 3 Relationship between HE and *H pylori* infection and blood ammonia

	HE (<i>n</i> = 217)	SHE (<i>n</i> = 55)	Cirrhotic (<i>n</i> = 62)	<i>P</i> value	χ ²
<i>H pylori</i> infection	74.4%	69.1%	53.2%	< 0.01	9.999
Child-Pugh class				< 0.01	29.154
A/B/C	27/100/90	9/30/16	22/33/7		
Ammonia concentration (μmol/L)	94.5 ± 75.6 ^b	59.9 ± 49.2	47.3 ± 33.5		

^b*P* < 0.01, *vs* SHE group (*t* = 4.117); *vs* cirrhotic (*t* = 1.601).

without HE (53.2%) (*P* < 0.05). Blood ammonia level differed significantly between cirrhotic patients with HE (94.5 ± 75.6 μmol/L), those with SHE (59.9 ± 49.2 μmol/L), or without HE 47.3 ± 33.5 μmol/L) (*P* < 0.05). Liver impairment of Child-Pugh class B and C in patients with HE and SHE were 87.6% and 83.6%, respectively. Child-Pugh class A and B accounted for 88.7% of cirrhotic patients without HE (Table 3).

Risk factors for HE

Through logistic multiple regression analysis, we found blood ammonia concentration (*P* = 0.000, OR = 4.701), Child-Pugh class (*P* = 0.000, OR = 3.416), *H pylori* infection (*P* = 0.007, OR = 2.113), gastrointestinal hemorrhage (*P* = 0.048, OR = 1.798), electrolyte disturbance (*P* = 0.045, OR = 1.875), and blood urea nitrogen (*P* = 0.041, OR = 1.854) were risk factors for HE. Sex, age, ascites, spontaneous bacteria peritonitis infection, hemoglobin, white blood count, platelet count and creatinine were not significantly associated with HE (Table 4).

DISCUSSION

Most currently available therapies for prevention of HE focus on reducing blood ammonia concentration^[14,15]. *H pylori* is known to produce copious amounts of ammonia due to its strong urease activity. Ammonia produced by *H pylori* has a role in the pathogenesis of hyperammonemia when this organism is widely distributed and present in large numbers in the stomach, particularly in the presence of liver cirrhosis^[16-19]. We did not find a significant difference in age, liver impairment and complication rate (upper gastrointestinal bleeding, hepatorenal syndrome and ascites) between *H pylori*-positive and -negative groups. However, blood ammonia concentration in *H pylori*-positive patients was significantly higher than that in *H pylori*-negative patients

Table 4 Risk factors for HE analyzed by logistic multiple regression

	<i>P</i> value	OR value	95% CI
Sex	0.341	0.751	0.416-1.354
Age	0.881	0.959	0.555-1.657
Etiology	0.125	1.564	0.883-2.769
<i>H pylori</i> infection	0.007	2.113	1.222-3.654
Blood ammonia level	0.000	4.701	2.773-7.970
Child-Pugh class	0.000	3.416	1.823-6.398
Ascites	0.277	1.395	0.765-2.541
Hemorrhage	0.048	1.798	1.004-3.218
Infections	0.934	1.027	0.546-1.932
Electrolyte disturbance	0.045	1.857	1.015-3.398
Leukocyte count	0.840	1.056	0.625-1.782
Hemoglobin	0.592	1.192	0.626-2.270
Platelet count	0.430	1.279	0.694-2.356
Creatinine	0.489	0.768	0.364-1.621
Blood urea nitrogen	0.041	1.854	1.025-3.353

(*P* < 0.01). This suggested that *H pylori* infection was associated with hyperammonemia in cirrhotic patients. It has previously been shown ammonia concentration in portal and venous blood significantly increased after the instillation of 10¹⁰ CFU/L *H pylori* in the stomach of cirrhotic rats^[20]. Oral administration of acetohydroxamic acid significantly reduced blood ammonia levels in cirrhotic patients with *H pylori* infection, compared with those without infection^[21]. We have previously reported that ammonia level in portal vein blood of cirrhotic patients with *H pylori* infection is significantly higher than that in patients without infection^[22].

In the present study, HE was more frequently observed in patients with *H pylori* infection than in those without (58.5% *vs* 30.6%, *P* < 0.01), which was consistent with that reported elsewhere^[23-25]. The hypothesis that *H pylori* infection plays a pathogenic role in HE was initially devised by Gubbins *et al*^[26]. In their study, seroprevalence for *H pylori* was detected in 78.6% of 117 alcoholic liver disease patients with HE, and in 62% of 71 patients without (*P* = 0.013). *H pylori* was detected only by serology, which has been reported to be inaccurate in cirrhotic patients. Therefore, the results of that study should be interpreted with caution. In a study of 55 cirrhotic patients, Dasani *et al*^[17] detected *H pylori* infection more frequently in those with HE compared with those without (67% *vs* 33%, *P* = 0.004). However, conflicting data are available in the literature. Several studies have shown that ammonia levels do not significantly differ between cirrhotic patients with and without *H pylori* infection, which suggests that although *H pylori* infection is able to generate ammonia in the stomach, the amount appears to be too small to affect arterial ammonia levels in patients with cirrhosis^[2,9,14,31]. The contribution of ammonia produced by *H pylori* to HE may depend on the number of bacteria and their distribution in the stomach, gastric pH, gastric membrane permeability to ammonia, liver impairment, and portal vein branch circulation. We suppose *H pylori* may increase blood ammonia concentration and induce HE when the bacterium is widely distributed in the stomach, and in the presence of severe liver impairment (Child-Pugh class B or C) with abundant portal vein branch circulation.

Through logistic multiple regression analysis, we found blood ammonia, Child-Pugh class, upper gastrointestinal bleeding, electrolyte disturbance, and urea nitrogen were significantly associated with HE. Dasani *et al.*^[17] have documented that risk factors associated with HE include older age ($P = 0.001$), lower albumin ($P = 0.001$), *H pylori* infection ($P = 0.004$), greater ascites score ($P = 0.01$), and greater Child-Pugh class ($P = 0.001$).

In view of the association of *H pylori* infection with hyperammonemia and HE, bacterium eradication may theoretically reduce ammonia concentration in cirrhotic patients^[27-29,32]. Ito *et al.*^[30] initially gave *H pylori* eradication therapy to cirrhotic patients, and found reduced ammonia concentration and recovery from HE after eradication, without relapse in the following 5 mo. In our study, blood ammonia concentration in *H pylori*-positive cirrhotic patients was significantly reduced by bacterium eradication ($P < 0.01$). HE rate significantly dropped to 34.1% after *H pylori* eradication ($P < 0.01$). However, several investigators have questioned whether the effect of eradication therapy on hyperammonemia is due to the non-specific effect of antibiotic therapy on the ammonia-producing gut flora. In Miyaji and Ito's study^[16], all patients were given lactulose, branched-chain enriched amino acid solution, low-protein diet, and kanamycin for 2 wk before *H pylori* eradication therapy, to reduce the effect of the gut flora on hyperammonemia. The blood ammonia concentration in patients with diffuse distribution of *H pylori* in the stomach was significantly reduced after bacterium eradication compared with the concentration after conventional treatment to reduce the gut flora. The ammonia concentration at 12 wk after eradication treatment was still significantly lower than that before. Therefore, eradication of *H pylori* to reduce bacterial ammonia production in the stomach is effective in patients with hyperammonemia with diffuse *H pylori* infection in the stomach, even after conventional therapy with a low-protein diet, antibiotics, lactulose and branched-chain enriched amino acid solution^[1,16,17]. *H pylori* eradication may be helpful for the treatment and prevention of HE. However, further studies are warranted to evaluate the arguments for and against the role of *H pylori* in the pathogenesis of HE.

COMMENTS

Background

Hepatic encephalopathy (HE) is a frequent complication of liver cirrhosis. Although the pathogenesis is unclear, ammonia is one of the key factors involved. Recently, it has been suggested *H pylori* contributes to hyperammonemia in cirrhotic patients and bacterium eradication decreases blood ammonia concentration. However, several other studies have shown ammonia levels do not significantly differ between cirrhotic patients with and without *H pylori* infection. The possible role of *H pylori* in the pathogenesis of HE merits further investigation.

Research frontiers

Recent research has focused on determining the relationship between *H pylori* infection, blood ammonia concentration and HE status in prospective and multicenter studies, and on investigating the effect of *H pylori* eradication on blood ammonia level and HE in cirrhotic patients.

Innovations and breakthroughs

We designed this prospective study to evaluate the effects of *H pylori* infection

and eradication on hyperammonemia and HE in 457 cirrhotic patients in five regions of Zhejiang Province, China. We observed blood ammonia concentration was significantly higher and HE was more frequent in patients with *H pylori* infection than in those without. Moreover, eradication of *H pylori* infection resulted in reduction in both blood ammonia concentration and frequency of HE.

Applications

H pylori infection is an important factor for inducing high blood ammonia concentration and HE in cirrhotic patients. *H pylori* eradication may be helpful for treatment and prevention of HE.

Terminology

SHE is characterized by normal, traditional clinical evaluation with definite and quantifiable neuropsychological defects.

Peer review

This study evaluated the relationship between *H pylori* infection, blood ammonia concentration and HE, and determined the effect of *H pylori* eradication on blood ammonia level and HE in cirrhotic patients. This study is of important clinical significance and should be of interest to readers of the journal.

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Changes of ghrelin following oral glucose tolerance test in obese children with insulin resistance

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Abstract

AIM: To characterize changes in ghrelin levels in response to oral glucose tolerance test (OGTT) and to correlate changes in ghrelin levels with changes in insulin and glucose following OGTT in Chinese obese children of Tanner I and II stage with insulin resistance.

METHODS: 22 obese children with insulin resistance state were divided into four groups according to their Tanner stage and gender: boys of Tanner I (BT-I), boys of Tanner II (BT-II), girls of Tanner I (GT-I), girls of Tanner II (GT-II). Ghrelin, insulin and glucose were measured at 0, 30, 60 and 120 min following OGTT. The control children with normal BMI were divided into control boys of Tanner I (CBT-I, $n = 6$), control boys of Tanner II (CBT-II, $n = 5$), control girls of Tanner I (CGT-I, $n = 6$), control girls of Tanner II (CGT-II, $n = 5$). Fasting serum ghrelin levels were analyzed.

RESULTS: Ghrelin levels were lower in obese groups. Ghrelin levels of control group decreased in Tanner II stage (CGT-I vs CGT-II $t = -4.703$, $P = 0.001$; CBT-I vs CBT-II $t = -4.794$, $P = 0.001$). Basal ghrelin levels in BT-II decreased more significantly than that in BT-I group ($t = 2.547$, $P = 0.029$). Ghrelin levels expressed a downward trend after OGTT among obese children. The decrease in ghrelin levels at 60 min with respect to basal values was 56.9% in BT-I. Ghrelin concentrations at 0 min correlated directly with glucose level at 0 min in BT-I ($r = 0.898$, $P = 0.015$). There wasn't a significant correlation of ghrelin changes with glucose changes and insulin changes during OGTT in obese children with insulin resistance.

CONCLUSION: In conclusion, in obese children with insulin resistance, ghrelin levels decreased with

advancing pubertal stage. Ghrelin secretion suppression following OGTT was influenced by gender and pubertal stage. Baseline ghrelin levels and ghrelin suppression after OGTT did not significantly correlate with the degree of insulin resistance and insulin sensitivity.

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Key words: Ghrelin; Oral glucose tolerance test; Insulin resistance; Obese children

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INTRODUCTION

Ghrelin is a novel GH-releasing peptide involved in the regulation of feeding behavior and energy homeostasis^[1]. Ghrelin secretion is up-regulated under conditions of negative energy balance and down-regulated in the setting of positive energy balance. Coexpression of GH secretagogue receptor and ghrelin in the pancreas suggests that this peptide is involved in glucose metabolism^[2]. Nutritional state is a determinant of plasma ghrelin in humans and rats^[3,4]. Endogenous ghrelin in islets acts on beta-cells to restrict glucose-induced insulin release at least partly via attenuation of Ca^{2+} signaling, and that this insulinostatic action may be implicated in the upward control of blood glucose^[5].

Though ghrelin concentrations in healthy children and adolescents and animals have been investigated^[6,7]. The role of ghrelin in childhood obesity, a state associated with hyperinsulinism and insulin resistance, is not fully understood. Previous reports demonstrated that plasma ghrelin levels decrease after oral glucose tolerance test (OGTT) in obese children and adults^[8-10]. To date, there no data are available on ghrelin levels after oral glucose administration in Chinese obese children. Similarly, ghrelin

levels with respect to puberty stage and obesity severity have never been investigated. Based on this background, the aims of the present study were to characterize changes in ghrelin levels in response to OGTT, and also to correlate changes in ghrelin levels with modifications in insulin and glucose in Chinese obese children of Tanner I and II stage with insulin resistance.

MATERIALS AND METHODS

Patients

The pubertal stages were determined by visual inspection, using Tanner's criteria^[11]. Children included in this study were ranging from Tanner I stage (aging 8.1 to 9.0 years) to Tanner II stage (aging 10.1 to 11.0 years) of pubertal development. Exclusion criteria were the presence of other endocrine disorders and the use of medication that could change the suggested laboratory evaluation at the time of the study. Age- and sex-specific body mass index (BMI) cut-off values can be used to identify adolescents with clustering of cardiovascular risk factors^[12-14]. The BMI of obese group varied from 25.4 to 29.7 kg/m². Twenty-two obese children with insulin resistance were divided into four groups according to their Tanner stage and gender: boys of Tanner I (BT-I, *n* = 6), boys of Tanner II (BT-II, *n* = 5), girls of Tanner I (GT-I, *n* = 6), girls of Tanner II (GT-II, *n* = 5). The control population was 22 healthy children with normal BMI (varied from 19.3 to 21.7 kg/m²), who were divided into control boys of Tanner I (CBT-I, *n* = 6), control boys of Tanner II (CBT-II, *n* = 5), control girls of Tanner I (CGT-I, *n* = 6), control girls of Tanner II (CGT-II, *n* = 5). Fasting serum ghrelin levels were analyzed in the control group, and the age of control group was matched to obese group in different puberty stage.

The human investigation committee of Zhejiang University School of Medicine approved the study. All subjects were informed about the purpose of this study and parents or guardians gave written consent.

Methods

All obese subjects were given 0.75 g/kg (maximum 75 g) of glucose solution orally after overnight fasting. Glucose was dissolved in about 200 mL of water and sipped over about 10 min to prevent nausea. Blood samples were collected at 0, 30, 60 and 120 min. Glucose concentrations were examined immediately after withdrawal. Blood samples were kept in chilled tubes containing EDTA (1 mg/mL) plus aprotinin (500 U/mL) for measuring ghrelin and insulin. The tubes were centrifuged at 3000 rpm/min and the plasma was stored at -80°C until assayed.

Insulin resistance was measured by the homeostasis model assessment (HOMA). The HOMA formulas are as follows:

- Homeostasis model assessment-insulin resistance index (HOMA-IR) = [fasting blood glucose (FBG, mmol/L) × fasting blood insulin (FINS, mIU/L)]/22.5. HOMA-IR ≥ 2.8 represents insulin resistance state^[13].
- HOMA insulin sensitivity index (HOMA-ISI) = 1/(FINS × FBG).

Plasma ghrelin levels were determined by a commercial

radioimmunoassay (Phonex Pharmaceutical. Inc, Belmont, CA, USA), using a polyclonal antibody that recognizes octanoylated and non- octanoylated ghrelin and ¹²⁵I-ghrelin as a tracer molecule. The intra- and interassay coefficients of variation were 5.0% and 10.7% respectively. Assay sensitivity was 12 pg/mL.

Plasma glucose concentrations were determined by the hexokinase method using an analyzer (Hitachi System 717; Roche Diagnostics, Basel, Switzerland).

Insulin was analyzed by Micro-particle enzyme immunoassay (IMMULITE system, Diagnostic Products Corporation, Los Angeles, USA).

Statistical analysis

The data were expressed either as mean ± SD or as 95% confidence intervals (95% CI). Normal distribution parameters were compared by independent-samples *t*-test or one-way ANOVA test. Non-normal distribution parameters were analyzed by Mann-Whitney *U* test. *P* < 0.05 was chosen as the level of significance. Linear regression analysis was performed to determine the overall interaction of different parameters, followed by partial correlation analysis.

RESULTS

The clinical features of obese I children

There were no differences in parameters such as insulin resistance, BMI, systolic blood pressure, *etc.*, among obese groups. A significant difference in insulin sensitivity was found (BT-I *vs* GT-II, *P* = 0.006; BT-I *vs* GT-II, *P* = 0.000; BT-I *vs* GT-II, *P* = 0.026, GT-II *vs* GT-I, *P* = 0.049) (Table 1).

Basal ghrelin levels in obese children and control group

Fasting serum ghrelin levels were analyzed. Compared with controls of the same gender and same Tanner stage, basal ghrelin levels were lower in obese groups, and there was significant difference in ghrelin levels between CGT-I group and GT-I group (*t* = 4.415, *P* = 0.02). Ghrelin levels of control group decreased in Tanner I stage (CGT-I *vs* CGT-II *t* = -4.703, *P* = 0.001; CBT-I *vs* CBT-II *t* = -4.794, *P* = 0.001). Basal ghrelin levels in BT-II decreased significantly than that in BT-I group (*t* = 2.547, *P* = 0.029). There were no differences in ghrelin levels between GT-I and GT-II (*t* = -1.743, *P* = 0.112) (Table 2).

Glucose, insulin and ghrelin levels after OGTT in obese children with insulin resistance

Ghrelin levels expressed a downward trend after OGTT among obese children (Table 3). Total ghrelin values (ghrelin 0 min plus ghrelin 30 min plus ghrelin 60 min plus ghrelin 120 min) were higher in BT-I than BT-II (*t* = 2.485, *P* = 0.032). At 0, 30, 60, 120 min during OGTT, GT-II group had no lower ghrelin levels than GT-I (*t* = 1.496, *P* = 0.169; *t* = -0.574, *P* = 0.580; *t* = -0.067, *P* = 0.968; *t* = 0.471, *P* = 0.649 respectively). The decrease in ghrelin levels at 60 min with respect to basal values was 56.9% in BT-I. This was the maximum ghrelin decrease following glucose administration, in parallel with maximum insulin levels. The maximum ghrelin decrease of GT-I occurred

Table 1 The clinical features of obese I children

	BT- I (n = 6)	BT- II (n = 5)	GT- I (n = 6)	GT- II (n = 5)
Age (yr)	9.30 ± 0.98	11.95 ± 0.99	8.72 ± 1.53	11.24 ± 1.08
Mean birth weight (kg)	3.59 ± 0.88	3.52 ± 0.38	3.61 ± 0.30	3.12 ± 0.13
Age of overweight beginning (yr)	5.43 ± 1.12	6.28 ± 2.92	4.05 ± 2.27	7.44 ± 3.87
Duration (yr)	4.67 ± 3.01	5.67 ± 3.27	4.67 ± 2.73	3.80 ± 3.70
BMI of patients (kg/m ²)	26.87 ± 1.52	27.75 ± 3.06	26.51 ± 1.66	28.62 ± 1.28
Systolic blood pressure (mmHg)	114.50 ± 16.03	132.33 ± 8.40	106.00 ± 7.87	116.00 ± 17.15
Diastolic blood pressure (mmHg)	66.50 ± 9.77	72.83 ± 12.45	71.40 ± 16.37	74.17 ± 5.63
Blood total cholesterol (mmol/L)	4.08 ± 0.38	4.07 ± 0.80	4.73 ± 0.73	3.87 ± 0.91
Blood triglyceride (mmol/L)	2.54 ± 2.33	1.11 ± 0.39	1.65 ± 0.47	1.22 ± 0.55
FBG/FINS-mmol/mIU	0.384 ± 0.119	0.395 ± 0.094	0.471 ± 0.108	0.218 ± 0.140
HOMA-IAI-mIU · mmol · l ⁻²				
Mean (LOG10)	-1.90 ± 0.38	-1.87 ± 0.24	-1.89 ± 0.51	-2.00 ± 0.10
HOMA-IR-mIU · mmol · l ⁻²				
Mean	4.82	3.72	4.48	4.52
95% CI	2.61-9.03	2.15-5.89	3.66-6.62	3.28-5.76
HOMA-IS-mIU/mmol				
Mean (LOG10)	2.04-0.33	1.82-0.30	2.22-0.34 ^c	2.58-0.06 ^{a,c,e}

BT- I : Boys of Tanner I ; BT- II : Boys of Tanner II ; GT- I : Girls of Tanner I; GT- II : Girls of Tanner II. Data are expressed as mean ± SD for Gaussian variables and as the median with lower and higher quartiles for non-Gaussian variables. ^a*P* < 0.05 *vs* BT- II ; ^c*P* < 0.05 *vs* BT- II ; ^e*P* < 0.05 *vs* GT- I .

at 30 min during OGTT, reaching approximately 39%, and it preceded the maximum increase in glucose levels. The maximum ghrelin decrease of BT- II and GT- II happened at 120 min, but it only reached 31% ± 10% and 9.8% ± 3% respectively. There were differences in ghrelin changes at 60 min from baseline levels between BT- I and BT- II (*F* = 8.402, *P* = 0.016), ghrelin value of GT- II at 60 min decreased more significantly than that of GT- I (*F* = 5.627, *P* = 0.041). However, the difference in terms of ghrelin changes between BT- II and GT- II happened at 30 min (*F* = 7.946, *P* = 0.020).

Ghrelin concentrations at 0 min during the oral glucose tolerance test correlated directly with glucose level at 0 min in BT- I (*r* = 0.898, *P* = 0.015) (Table 4). Although ghrelin values varied during OGTT, we could not demonstrate a significant correlation of ghrelin changes with glucose changes and insulin changes during OGTT in obese children with insulin resistance.

DISCUSSION

Ghrelin plays a role in meal initiation and satiety in an inverse pattern to that of insulin^[2,3]. Previous reports demonstrated that ghrelin levels were significantly decreased in obese children^[8,15]. However, the secretory dynamics of ghrelin have not been characterized in obese children with insulin resistance. In this study, obese children with insulin resistance were divided into different groups by gender and pubertal stage to observe the effects of gender and puberty on ghrelin levels. In control children, basal ghrelin levels of Tanner II group were lower than those of Tanner I group. In obese children with insulin resistance, basal ghrelin levels in BT- II group decreased significantly than that in BT- I group, however, there were no differences in ghrelin levels between GT- I and GT- II. This result indicates that basal ghrelin levels differ depending upon the pubertal stage and gender. The increase in sexual hormones is associated with a marked decline in circulating levels of ghrelin in

Table 2 Basal ghrelin levels in obese children and control group (pg/mL)

	Boys		Girls	
	Tanner I	Tanner II	Tanner I	Tanner II
Obese children	1148.2	464.9 ^a	1043.6	429.3 ^c
	872.3-1424.2	220.2-809.6	772.3-1220.3	182.6-1027.4
Control children	1009.6	244.5 ^c	412.9 ^a	222
	741.4-1777.7	165.7-323.3	134.8-691.1	113.1-359.0

Data are expressed as mean (95% CI). ^a*P* < 0.05 *vs* control group of the same gender and same Tanner stage; ^c*P* < 0.05 *vs* subgroup of the same gender and different Tanner stage within the control group; ^e*P* < 0.05 *vs* subgroup of the same gender and different Tanner stage within the obese group.

boys, serum testosterone are the major determinants of serum ghrelin^[16]. Different estrogen and testosterone levels influence the body weight homeostasis of growth hormone secretagogue receptor (GHSR) -/- mice, which lack the orexigenic ghrelin signaling^[17-19]. Contrary to what is expected in physiologic puberty, where ghrelin is progressively reduced, in central precocious puberty (CPP), ghrelin secretion seems to be independent from pubertal development. Concomitant estrogen suppression during treatment may play a potential role in the regulation of ghrelin secretion in CPP girls^[20]. With advancing pubertal stages, ghrelin levels may be prone to be influenced by sexual hormones and growth hormone, so they display gender differences.

The rapid fall in plasma ghrelin concentration after glucose load suggests its involvement in the control of appetite and in the regulation of energy homeostasis^[21]. The maximum decrease in ghrelin levels happened at 60 min in simple obesity adults (BMI, 26.3-40.5)^[22,23]. OGTT-induced absolute suppression in ghrelin was approximately 50% less in overweight versus normal weight children, resulting in a similar percent suppression from baseline in the two groups^[24,25]. In this study, the entity of ghrelin suppression during OGTT differed with gender and pubertal stage in obese children with insulin resistance.

Table 3 Glucose, insulin and ghrelin level after OGTT in obese children

Group	Parameters	0 min	30 min	60 min	120 min
BT- I	Glucose-mmol/L				
	Mean	4.8	6.6	6.4 ^a	5.7 ^{c,e}
	95% CI	4.6-5.1	5.8-7.4 ^a	5.1-7.6	4.2-7.2
	Insulin-mIU/L				
	Mean	21.9	97.9 ^a	135.3 ^a	103.3 ^{c,e}
	95% CI	3.9-40.0	21.5-174.3	14.0-284.4	57.1-263.6
BT- II	Ghrelin-pg/mL				
	Mean	1009.6	505.2	353.3 ^a	360.6
	95% CI	241.4-1777.7	90.1-920.3	65.1-771.6	49-770.3
	Glucose-mmol/L				
	Mean	5.1	8.0 ^a	7.5 ^a	4.3 ^{c,e}
	95% CI	4.7-5.3	7.2-8.9	6.3-8.6	3.8-4.8
BT- II	Insulin-mIU/L				
	Mean	16.6	114.2 ^a	85.9 ^a	14.1 ^{c,e}
	95% CI	6.6-26.7	65.6-152.8	32.8-139.0	9.9-18.4
	Ghrelin-pg/mL				
	Mean	244.5	192.6	230.9	154.9
	95% CI	165.7-323.3	148.2-231.7	93.1-368.6	169.6-213.9
GT- I	Glucose-mmol/L				
	Mean	5.4	6.1	5.9	6.4
	95% CI	4.5-6.3	5.1-7.1	4.8-7.1	4.6-8.1
	Insulin-mIU/L				
	Mean	26.4	62.1	55.9	32.5
	95% CI	14.7-67.6	14.7-138.9	21.2-133.1	4.0-68.9
GT- I	ghrelin-pg/mL				
	Mean	412.9	252.3	310	266.2
	95% CI	134.8-691.1	77.9-392.8	132.5-487.6	55.4-476.9
GT- II	Glucose-mmol/L				
	Mean	4.7	7.6	7.1	5.2 ^{c,e}
	95% CI	4.3-5.1	6.5-8.7 ^a	5.7-8.5a	3.4-7.0
	Insulin-mIU/L				
	Mean	21.7	109.4 ^a	81.3	24.6 ^c
	95% CI	17.3-26.1	28.1-190.8	9.5-153.2	18.9-30.3
GT- II	Ghrelin-pg/mL				
	Mean	222	309.4	316.9	202.2
	95% CI	85.1-359.0	96.1-714.8	109.4-524.4	21.8-392.7

^a*P* < 0.05 vs 0 min in the same group; ^c*P* < 0.05 vs 30 min; ^e*P* < 0.05 vs 60 min.

The maximum decrease in ghrelin levels was about 57%, at 60 min in Tanner I boys. However, the maximum ghrelin decrease of GT- I occurred at 30 min, reaching approximately 39%. The maximum ghrelin decrease of BT- II and GT- II groups happened later, and the entity of the decrease lessened. This result demonstrated that the ghrelin secretion pattern of obese children with insulin resistance was different from simple obesity adults and overweight children. Gender differences in ghrelin suppression after OGTT in obese children with insulin resistance were also noted; further studies are needed to elucidate the mechanism underlying this phenomenon.

Fasting ghrelin levels were mainly influenced by insulin sensitivity independently from adiposity^[26]. Ghrelin is substantially decreased during pregnancy, but glucose-induced ghrelin suppression is preserved at a lower level. There is apparently no relation to the degree of insulin resistance^[27,28]. Plasma ghrelin concentrations in obese children with insulin resistance were lower than those of control children in our study, which were in accordance with previous reports. In this study, the correlation between baseline ghrelin levels and basic factors involved in glucose homeostasis were further analyzed, Baseline ghrelin levels of obese children with insulin resistance have

Table 4 The correlation of baseline ghrelin levels with some baseline indexes involved in glucose homeostasis

Group	Vs FBG r (P)	Vs FINS r (P)	Vs FBG/FINS r (P)	Vs HOMA-IAI r (P)	Vs IR r (P)	Vs IS r (P)	Vs BMI r (P)
BT- I	0.898 (0.015) ^a	0.488 (0.326)	0.35 (0.947)	0.297 (0.568)	0.552 (0.269)	0.435 (0.338)	0.737 (0.095)
BT- II	0.045 (0.859)	0.1 (0.693)	0.929 (0.007) ^a	0.896 (0.016) ^a	0.772 (0.072)	0.85 (0.032) ^a	0.672 (0.114)
GT- I	0.074 (0.889)	0.194 (0.062)	0.25 (0.633)	0.027 (0.959)	0.206 (0.296)	0.018 (0.973)	0.065 (0.903)
GT- II	0.135 (0.829)	0.551 (0.336)	0.668 (0.218)	0.466 (0.299)	0.419 (0.482)	0.301 (0.622)	0.557 (0.330)

^a*P* < 0.05.

not correlations with some clinic indexes as reported in patients with type 2 diabetes and overweight children^[29,30]. Baseline ghrelin levels correlated with insulin sensitivity and β -cell function only in BT- II group. Baseline ghrelin concentrations in BT- I group correlated with fasting blood glucose. There were no relationships between baseline ghrelin levels and baseline glucose, insulin concentrations and insulin sensitivity in BT- II and GT- II

groups. There was no correlation between baseline ghrelin and dynamic glucose and insulin data.

Alterations in ghrelin suppression in overweight children may be yet another manifestation of the insulin resistance of obesity^[26]. Ghrelin parameters were inversely associated with fasting insulin, HOMA-IR in adolescent girls with anorexia nervosa^[31]. However, we could not demonstrate a significant correlation between ghrelin level changes, glucose and insulin concentrations after OGTT in obese children with insulin resistance. Ghrelin suppression after OGTT is modulated by insulin sensitivity. Whether ghrelin suppression in obese children with insulin resistance is a manifestation or an outcome of insulin resistance requires additional investigation.

In conclusion, in obese children with insulin resistance, ghrelin levels decreased with advancing pubertal stage. Ghrelin secretion suppression following OGTT was influenced by gender and pubertal stage. Baseline ghrelin levels and ghrelin suppression after OGTT did not significantly correlate with the degree of insulin resistance and insulin sensitivity.

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COMMENTS

Background

Ghrelin plays a role in the regulation of energy balance and attenuates leptin-induced reduction in food intake and body weight. Ghrelin levels were found decreased in obese individuals and influenced by the pubertal stage. However, the relationship between ghrelin secretion and insulin resistance, pubertal stage are not completely understood.

Research frontiers

Obesity increases the risk of developing type 2 diabetes, hypertension, stroke, and heart attack. Insulin resistance has a central role in above chronic diseases. A reciprocal relationship exists between ghrelin and insulin, suggesting that ghrelin regulates glucose homeostasis. However, the secretory dynamics of ghrelin have not been characterized in obese children with insulin resistance.

Innovations and breakthroughs

In obese children with insulin resistance, ghrelin levels decreased with advancing pubertal stage. Ghrelin secretion was influenced by gender and its suppression following OGTT differed with gender and pubertal stage.

Applications

Taken gender and puberty into consideration, alterations in ghrelin suppression in obese children may be another manifestation of the insulin resistance.

Terminology

Tanner's pubertal staging of the secondary sexual characteristics that identify pubertal progression are a cornerstone for both clinicians and those involved in clinical research of children and adolescents. This staging has served as the foundation for the study and understanding of the maturation of the hypothalamic-pituitary-gonadal axis, adrenarche, and the physiological processes that initiate and facilitate progression of sexual maturation. According to Tanner's description, progression of sexual maturation is divided into Tanner's stage I, II, III, IV and V stage.

Peer review

This study investigated plasma ghrelin changes in response to OGTT, and also

to correlate changes in ghrelin levels with modifications in insulin and glucose in Chinese obese children of Tanner and stage with insulin resistance. It is of particular importance to obese children with insulin resistance.

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Comparative analysis of common *CFTR* polymorphisms poly-T, TG-repeats and M470V in a healthy Chinese population

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Abstract

AIM: To investigate the three important cystic fibrosis transmembrane conductance regulator (*CFTR*) haplotypes poly-T, TG-repeats and the M470V polymorphisms in the Chinese population, and to compare their distribution with that in Caucasians and other Asian populations.

METHODS: Genomic DNA was extracted from blood leukocytes. Exons 9 and 10 of the *CFTR* gene were obtained through polymerase chain reaction (PCR). Exon 9 DNA sequences were directly detected by an automated sequencer and poly-T and TG-repeats were identified by direct sequence analysis. Pure exon 10 PCR-amplified products were digested by *Hph* I restriction enzyme and the M470V mutation was detected by the AGE photos of digestion products.

RESULTS: T7 was the most common (93.6%) haplotype and the (TG)11 frequency of 57.2% and (TG)12 frequency of 40.9% were dominant haplotypes in the junction of intron 8 (IVS-8) and exon 9. The frequency of T5 was 3.8% and all T5 allele tracts (10 alleles) were joined with (TG)12. Four new alleles of T6 (1.5%) were found in three healthy individuals. In exon 10, the V allele (56.1%) was slightly more frequent than the M allele (43.9%), and the M/V (45.5%) was the dominant genotype in these individuals. The three major haplotypes T7-(TG)11-V470, T7-(TG)12-M470 and T7-TG11-M470 were related to nearly 86.0% of the population.

CONCLUSION: The polymorphisms of poly-T, TG-repeats, and M470V distribution were similar to those in other East Asians, but they had marked differences in frequency from those single haplotype polymorphisms or linkage haplotypes in Caucasians. Thus, they may be able to explain the low incidence of CF and CF-like diseases in Asians.

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Key words: Cystic fibrosis transmembrane conductance regulator gene; Gene polymorphism; Poly-T; TG-repeats; M470V

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INTRODUCTION

The cystic fibrosis transmembrane conductance regulator (*CFTR*) gene on chromosome 7q31 spans approximately 250 kb of DNA and encodes 27 exons encodes^[1]. The *CFTR* gene encodes a cAMP- and ATP-dependent chloride channel that is present in the apical membrane of the epithelial cells that line most exocrine glands^[2]. Phosphorylation of the regulatory domain by protein kinase A, followed by binding and hydrolysis of ATP at both nucleotide-binding domains, regulates the transport of chloride ions through the channel^[3]. Absence, reduced levels, or malfunction of the *CFTR* protein results in cystic fibrosis (CF), and CF-like diseases such as congenital bilateral absence of the vas deferens (CBAVD)^[4,5], bronchiectasis^[6] and chronic pancreatitis^[7]. Since the discovery of the *CFTR* gene, more than 1000 mutations and 200 polymorphisms have been identified^[8]. CF is one of the most common autosomal recessive disorders in Caucasians, with an incidence of approximately 1 in 2500 Caucasian births and a carrier frequency of approximately 1 in 25. However, in Asians, the prevalence of CF is very low, with an incidence of approximately 1 in 100000, and in particular, the severe mutations, such as $\Delta F508$, G542X and N1303K, are rarely found in Asians. Previous studies have demonstrated that polymorphisms outside the *CFTR* gene^[9,10], as well as within the gene, may affect transcription or function of the *CFTR* protein and modify the phenotype of some CF mutations. It has been mentioned that poly-T, TG-repeats and M470V polymorphisms play a role in the development of CF-like diseases. The poly-T tract located at the junction of intron

8 (IVS-8) and exon 9 influence transcription, and thereby reduce the amount of normal CFTR protein. The number of T residues present, five, seven or nine, affects the splicing efficiency of exon 9. If the T5 allele is present, a proportion of CFTR transcripts will lack exon 9, which produces a non-functional protein and variable CF symptoms^[11]. The TG-repeats, 5' of the poly-T, also influence splicing of exon 9^[12], and when present on the same allele as a 5T repeat, the longer the TG-repeats, the higher the proportion of CFTR transcripts that will lack exon 9. On the other hand, the M470V polymorphism on exon 10 affects the intrinsic chloride activity, and thereby affects the function of the CFTR protein^[12,13].

Although mutations and polymorphisms of CFTR have been extensively studied in Western populations, their importance is less well studied in East Asia because of the rare presentation of classical CF. There are just a few data on CFTR in Asia, especially in China. No reports on CFTR genetic background among the normal Chinese population have been published, except for sporadic reports on CFTR mutations in CF-like patients. To explore polymorphic backgrounds of CFTR in the Chinese population, we analyzed polymorphisms of poly-T, TG repeats and M470V in 132 healthy individuals among the general population in Jiangsu Province.

MATERIALS AND METHODS

Subjects

A total of 132 healthy unrelated subjects were randomly selected from the population of Jiangsu Province (78 males, 54 females; mean age 44 years, range 16-85 years). Four milliliters of blood were collected for genotyping. The blood was mixed with EDTA and stored at -80°C.

DNA analysis

Genomic DNA was extracted from blood leukocytes using the QiaAmp DNA Blood Mini kit (Qiagen, Hilden, Germany). Polymerase chain reaction (PCR) was carried out using Ex Taq Polymerase (Takara, Japan). The PCR used a GeneAmp PCR system (Model PTC-200, Bio-Red, Foster City, CA, USA). Cycling for both reactions was performed as follows: 94°C for 5 min for preheating, 35 cycles at 94°C for 30 s, 60°C for 60 s, and 72°C for 30 s, followed by one cycle at 72°C for 10 min for extension. The oligonucleotide primers used were: Intron 8 and exon 9 junction, sense 5'-CCATGTGCTTTTCAAACCTAAT TGT-3', anti-sense 5'-TAAAGTTATTGAATGCTCGC CATG-3'; and exon 10, sense 5'-TTGTGCATAGCAG AGTACCTGAAA-3', anti-sense 5'-GCTTCTTAAAGC ATAGGTCATGTG-3'. The sequences of exon 9 PCR-amplified products were sequenced by Shanghai Invitrogen Company using an automated sequencer (ABI 737). Exon 10 PCR-amplified products were purified using the High Pure PCR Product Purification Kit (Roche Diagnostics, Mannheim, Germany). The M470V mutation was detected by *Hpa*II restriction enzyme digestion.

Haplotype analysis

Haplotypes consisting of three loci were investigated,

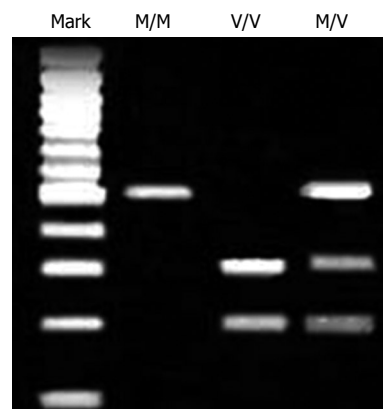


Figure 1 M470V polymorphism in exon 10.

Table 1 Allele frequency of poly-T tract

Number (n)	Number (frequencies) of poly-T tract			
	T5	T6	T7	T9
2n = 264	10 (0.0379)	4 (0.0152)	247 (0.9356)	3 (0.0114)

that is, poly-T, TG-repeats, and the M470V. The M470V polymorphisms were estimated by the AGE photos of pure PCR products after *Hpa*II restriction enzyme digestion (Figure 1). Poly-T and TG-repeats are continuous in sequence, hence their haplotypes were identified by direct sequence analysis (Figure 2). The frequency of each haplotype of TG-repeats and M470V (P_m) was estimated by the following equation derived from the Hardy-Weinberg law: $(P_1 + P_2 + P_3 + P_m)^2 = 1$, where $P_1 + P_2 + P_3 + P_m = 1$, and $P_1^2, P_2^2, P_3^2, P_m^2$ are the frequencies of homozygous for either locus or both loci.

RESULTS

Poly-T

T7 was the most common haplotype (93.6%), hence, T7/T7 was a dominant genotype in Chinese individuals (Table 1). Four alleles of T6 were newly found in the normal subjects. Ten alleles of T5 with a frequency of 3.8% were found. The T9 alleles were very rare at just 1.1%. The sequence analysis indicated that four alleles of T6 probably resulted from a T deletion from T7.

TG-repeats

(TG)11 and (TG)12 were the dominant haplotypes in the Chinese population, with a frequency of 57.2 and 40.9%, respectively. The frequency distribution of genotypes (TG)11/(TG)11, (TG)11/(TG)12 and (TG)12/(TG)12 was 46.2, 21.2 and 29.6%, respectively (Table 2). The relative ratio of the (TG)11/(TG)11, (TG)11/(TG)12 and (TG)12/(TG)12 was roughly 2:1:1.

M470V

The V allele was slightly more frequent than the M allele in the Chinese population, and the frequencies were 56.1 and 43.9%, respectively. The dominant genotype was M/V, followed by V/V and M/M (Table 3).

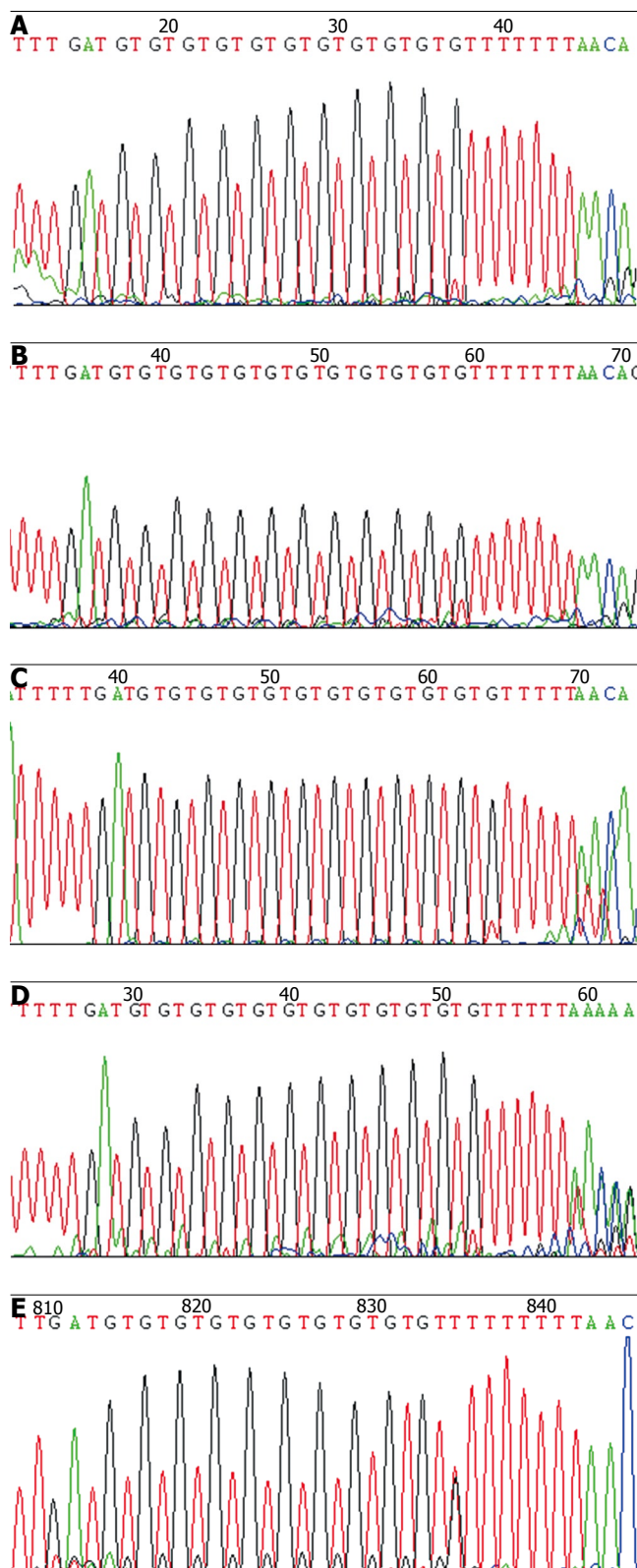


Figure 2 The polymorphisms of TG-repeats and Poly-T haplotype in IVS-8. **A:** TG11-T7; **B:** TG12-T7; **C:** TG12-T5; **D:** TG12-T6; **E:** TG10-T9.

Three-locus haplotype analysis

T7-TG11-V470 was the dominant linkage haplotype in the Chinese population, and the frequency was 47.4%, which was almost half of all the types of linkage. Other frequencies were: T7-TG12-M470, 29.2%; T7-TG11-M470, 9.9%; and T7-TG11-M470, 6.8%, and others

Table 2 Allele frequency of TG-repeat polymorphisms

Number (2 <i>n</i>)	Number (frequencies) of TG-repeats				
	TG10	TG11	TG12	TG13	
264	3 (0.0114)	151 (0.572)	108 (0.4091)	2 (0.0076)	
	TG-repeat number (frequencies) in individuals with alleles				
	11/11	11/12	12/12	11/13	10/12
132	61 (0.4621)	28 (0.2121)	39 (0.2955)	2 (0.0152)	2 (0.0152)

Table 3 Genotypes and allele frequencies at M470V polymorphic site

Number ($2n$)	Number (frequencies) of individuals with genotypes			Number (frequencies) of individuals with alleles	
	MM	MV	VV	M	V
264	28 (0.2121)	60 (0.4545)	44 (0.3333)	116 (0.4394)	148 (0.5606)

Table 4 Linkage of poly-T, TG-repeats and M470V

Linkage haplotypes	$2n$ (frequencies) ($2n = 264$)
T7-TG11-V470	125 (0.4735)
T7-TG12-M470	77 (0.2917)
T7-TG11-M470	26 (0.0985)
T7-TG12-V470	18 (0.0682)
T5-TG12-M470	8 (0.0303)
T5-TG12-V470	2 (0.0076)
T7-TG13-M470	2 (0.0076)
T9-TG10-M470	1 (0.0038)
T9-TG10-V470	1 (0.0038)
T6-TG12-M470	2 (0.0076)
T6-TG12-V470	1 (0.0038)
T6-TG11-V470	1 (0.0038)

were very rare (Table 4). The T5 alleles were all linked with (TG)12, and its distribution ratio at M470 and V470 loci was 4:1.

DISCUSSION

The present study is believed to be the first comprehensive report on the functional polymorphisms of CFTR in the Chinese population. Analysis of three polymorphic loci with frequent alleles in the general population showed the poly-T, TG-repeats and M470V distributions were similar to those reported for other East Asians^[14-16]. T7 was the most common haplotype (93.6%), and (TG)11 and (TG)12 were the dominant haplotypes in the junction of intron 8 (IVS-8) and exon 9. In exon 10, the V allele was slightly more frequent than the M allele, and the M/V genotype was the dominant genotype. The three major haplotypes T7- (TG)11-V470, T7- (TG)12- M470 and T7-TG11-M470 were found in nearly 86.0% of the population.

Similar to other populations, the T7 allele was the most common haplotype (93.6%) in IVS-8, and T7/T7 was the dominant genotype in Chinese individuals. The T6 allele, in addition to the well-known T5, T7 and T9 alleles, was

found. It has not been reported in Caucasians, but it has been reported in Asians, including Vietnamese^[14], Japanese^[15] and Koreans^[16]. However, its functional transcript and disease association is uncertain at this time. Four T6 alleles (1.5%) were found in three normal individuals, and the frequency was higher than that in Japanese (1.2%)^[15] and Vietnamese (0.1%)^[14]. The T9 allele was found in three alleles, and its frequency of 1.1% was higher than that in Japanese (0.6%-1.0%)^[14,15], Vietnamese (0.6%)^[14] and Koreans (0.52%)^[16] but lower than in Caucasians (7.0%)^[14]. It is known that polymorphisms in IVS-8 Tn tract affect the RNA splicing of exon 9, and the T9 allele is associated with the most efficient usage of the IVS-8 splice acceptor site^[11]. This efficiency decreases with shorter poly-T tract T5, which results in a lower than normal level of full-length CFTR mRNA, and presumably a decrease in mature, functional CFTR protein. T5 may be the most common atypical CF mutation worldwide. Prior studies have demonstrated that some individuals who carry T5 with a severe CF-causing mutation may have non-classic CF; others may have male infertility due to CBAVD^[5,17-20], lung disease such as bronchiectasis^[6,21,22] and chronic pancreatitis^[23,24]; and approximately 40% may be healthy and fertile as a consequence of incomplete penetrance^[5,17]. In our study, the frequency of the T5 allele in the Chinese population was 3.8%, which is similar to the Vietnamese (3.7%)^[14], but lower than that in Caucasians (7.0%)^[5,13], and higher than in Japanese (0.6-1.0%)^[14,15] and Koreans (1.7%)^[16].

The main TG-repeat was (TG)11/11, with 61 (46.21%) individuals having the (TG)11/11 haplotype. (TG)11 was the dominant haplotype in the Chinese population, with a high frequency of 57.2%. This was higher than in Vietnamese (41%)^[14] and Japanese (51%-54%)^[14,15], but lower than in Caucasians (67%)^[14]. The main dominant haplotypes were (TG)11 and (TG)12 in the Chinese population, as well as other Asian populations, however, in Caucasians, after the (TG)11 haplotype, the most common was the (TG)10 haplotype^[14,25]. As previously reported, the TG-repeats that join with poly-T tracts also influence splicing of exon 9^[12], and when present on the same allele as a T5 tract, the longer the TG-repeats, the higher the proportion of CFTR transcripts that will lack exon 9. T5 allele adjacent to either (TG)12 or (TG)13 repeats is more likely to exhibit an abnormal phenotype than T5 adjacent to (TG)11^[26]. In our study, all T5 allele tracts (10 alleles) were joined with (TG)12 repeats. The TG repeat number also exerts an effect on a T7 background. Compared with (TG)10 allele, TG (11) increases almost threefold the proportion of CFTR transcripts that lack exon 9^[12].

At the M470V locus on exon 10, similar to other Asian populations and Caucasians, the V allele (56.1%) was more frequent than the M allele (43.9%) in the Chinese population. The M470V polymorphism is a missense mutation caused by a particular amino acid alteration in the exon 10 M470V locus. It has been shown the M470 allele causes a delay in CFTR protein maturation and gives rise to a chloride channel with an increased probability of being open, compared with the V470 CFTR protein^[12]. It has also been shown that the variability of European random CFTR genes is almost completely restricted to those who carry the M allele of the M470V polymorphic site^[27].

Table 5 Frequencies of TG-repeats and M470V haplotypes

M470V	(TG) n	n/frequencies (n = 132)
V/V	11/11	35 (0.2625)
M/M	12/12	27 (0.2045)
M/V	11/11	26 (0.1970)
M/V	11/12	20 (0.1515)
M/V	12/12	11 (0.0833)
V/V	11/12	8 (0.0606)
M/V	11/13	2 (0.0152)
V/V	12/12	1 (0.0076)
M/M	10/12	1 (0.0076)
M/V	10/12	1 (0.0076)

However, interestingly, the M allele has been reported for some CF mutations, and particularly for $\Delta F508$ ^[28-30], most mutations have been found to be associated with the M470 allele, while the V470 allele shows an extended haplotype homozygosity^[27,31,32].

The T7 allele tracts were combined with (TG)11 and (TG)12 repeats, but the T7-(TG)10 haplotype was not found in our study. In the T7 background, the (TG)11/(TG)11-V/V, (TG)12/(TG)12-M/M, (TG)11/(TG)11-M/V and (TG)11/(TG)12-M/V were the four main haplotypes and almost equally distributed in the Chinese population (Table 5). We found that TG-repeats and M470V had a linkage distribution in that V/V was combined with (TG)11/(TG)11, and M/M was combined with (TG)12/(TG)12 haplotype. The major haplotypes were T7-(TG)11-V470 and T7-(TG)12-M470, similar to Japanese and Vietnamese, but in the Chinese and Japanese, the main haplotype was T7-TG11-V470. Conversely, T7-TG12-M470V was the main haplotype in Vietnamese. However, in Caucasians, two main haplotypes, T7-(TG)11-V470 and T7-(TG)10-M470 are predominant^[14,25]. As previously reported, TG-repeats also influence the function of CFTR protein, and longer TG repeats increase the proportion of CFTR transcripts that lack exon 9^[12]. Therefore, the two major (TG)11/(TG)12-bearing haplotypes may have a corresponding low CFTR activity in Asian populations compared with the dominant (TG)11/(TG)10-bearing haplotypes in Caucasians. This may explain the low incidence of CF and CF-like diseases in Asians.

This report provides evidence for a poly-T, TG-repeat and M470V haplotype background in the Chinese population. We found polymorphisms of poly-T, TG-repeats and M470V were similarly distributed in other East Asians, and have marked differences from the frequencies of single haplotype polymorphisms or linkage haplotypes in Caucasians. Further study of the relationship between polymorphisms of poly-T, TG repeats and M470V haplotypes in CF and CF-like diseases in the Chinese population should be undertaken.

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COMMENTS

Background

The cystic fibrosis transmembrane conductance regulator (CFTR) gene encodes a cAMP- and ATP-dependent chloride channel that is present in the apical membrane of the epithelial cells that line most exocrine glands. Absence, reduced levels, or malfunction of the CFTR protein results in CF and CF-like diseases, such as CBAVD, bronchiectasis and chronic pancreatitis. It has been reported that poly-T, TG-repeats and M470V polymorphisms play a role in the development of CF-like diseases. The present study is believed to be the first comprehensive report on functional polymorphisms of CFTR in the Chinese population.

Research frontiers

The CFTR gene is a cAMP- and ATP-dependent chloride ion transport channel. The three haplotypes poly-T, TG-repeats and M470V polymorphisms play a role in the development of CF-like diseases. Although mutations and polymorphisms of CFTR have been extensively studied in Western populations, their importance is less well-established in East Asia. The present study is believed to be the first comprehensive report on functional polymorphisms of CFTR in the Chinese population. This study provides evidence for poly-T, TG-repeat and M470V haplotype backgrounds in the Chinese population.

Innovations and breakthroughs

There are just a few data on CFTR in Asia, especially in China. No reports on CFTR genetic background among the normal Chinese population have been published, except for sporadic reports on CFTR mutations in CF-like patients. The present study is believed to be the first comprehensive report on functional polymorphisms of CFTR in the Chinese population. This study provides evidence for poly-T, TG-repeat and M470V haplotype backgrounds in the Chinese population.

Applications

Comparative analysis of common CFTR polymorphisms in poly-T, TG-repeats and M470V in the healthy Chinese population sheds light on the situation of CFTR gene mutations and polymorphisms in the Chinese population. This study provides a polymorphic background of CFTR in the Chinese population, and helps to understand CF-like diseases such as CBAVD, bronchiectasis and chronic pancreatitis in China.

Terminology

CFTR gene on chromosome 7q31 spans approximately 250 kb of DNA and encodes 27 exons. The CFTR gene encodes a cAMP- and ATP-dependent chloride channel that is present in the apical membrane of the epithelial cells which line most exocrine glands.

Peer review

This interesting study investigated three important CFTR haplotypes, poly-T, TG-repeats and M470V polymorphisms in the Chinese population. The results may explain the low incidence of CF and CF-like diseases in Asians.

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Prognostic significance of S100A4 and vascular endothelial growth factor expression in pancreatic cancer

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Abstract

AIM: To investigate the expression of vascular endothelial growth factor (VEGF) and calcium-binding protein S100A4 in pancreatic cancer and their relationship to the clinicopathological parameters and prognosis of pancreatic cancer.

METHODS: Expression status of VEGF and S100A4 was examined in 62 surgical specimens of primary pancreatic cancer by immunohistochemistry. Correlation between the expression of VEGF and S100A4 and clinicopathological parameters was analyzed.

RESULTS: Thirty-eight of 62 (61.3%) specimens of primary pancreatic cancer were positive for S100A4. Thirty-seven (59.7%) specimens showed positive expression of VEGF. The positive correlation between S100A4 and VEGF expression was significant in cancer tissues ($P < 0.001$). S100A4 expression was significantly correlated with tumor size, TNM stage and poorer prognosis. VEGF expression had a significant correlation with poorer prognosis. The prognosis of 17 S100A4- and VEGF-negative cancer patients was significantly better than that of other patients ($P < 0.05$). Distant metastasis ($P = 0.001$), S100A4- ($P = 0.008$) and VEGF-positive expression ($P = 0.016$) were significantly independent prognostic predictors ($P < 0.05$).

CONCLUSION: Over-expression of S100A4 and VEGF plays an important role in the development of pancreatic cancer. Combined examination of the two molecules might be useful in evaluating the outcome of patients with pancreatic cancer.

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Key words: Pancreatic cancer; Prognosis; S100A4; Vascular endothelial growth factor; Immunohistochemistry

INTRODUCTION

Each year there are more than 170 000 new cases of pancreatic cancer in the world. Pancreatic cancer accounts for up to 2.1% of all cancer cases and is the 5th leading cause of cancer death. The survival rate of patients at all stages of the disease is poor. The overall median survival time is 3-5 mo with a 12 mo-survival rate of 10% and a 5-year survival rate less than 5%^[1]. Because of lacking of methods for the early diagnosis and limited knowledge on the biological features of pancreatic cancer, the majority of patients are not diagnosed properly until the advanced stage^[2].

The S100 protein family is a large family of soluble calcium-binding proteins, first isolated from bovine brain by Moore in 1965^[3]. The S100 family members are involved in a variety of physiological functions, such as cell motility, cell proliferation and differentiation, cell cycle control, regulation of enzyme activity, and calcium-dependent transcriptional regulation^[4-7]. The S100A4 protein, which was once named as mts1 or p9Ka, belongs to the S100 family and is classified as a 'metastasis-related gene'^[8]. It was reported that over-expression of S100A4 is significantly correlated with tumor invasion and metastasis^[9-11]. A number of studies suggested that over-expression of S100A4 is correlated with poor clinical outcomes in a variety of human cancers, such as bladder, colorectal, ovarian, and esophageal carcinoma^[12-15]. VEGF plays an important role in tumor angiogenesis and correlates significantly with tumor invasion and metastasis^[16]. Elevated levels of VEGF correlate with a poor prognosis of various cancers, including pancreatic cancer^[17,18].

This study was to examine the expression status of S100A4 and VEGF in 62 surgical specimens of primary pancreatic carcinoma by immunohistochemistry and study the role of these two molecules in progression and metastasis of pancreatic cancer.

MATERIALS AND METHODS

Specimens

Specimens obtained from 62 patients (36 males, 26 females) with primary pancreatic cancer admitted to Department of Surgery, 6th Affiliated Hospital of Shanghai Jiaotong University in 2002-2005, were formalin-fixed and paraffin-embedded. The age of these patients ranged 30-84 years (mean age of 64.8 years). All cases were diagnosed as primary pancreatic ductal adenocarcinoma by histopathology (well differentiated in 17 cases, moderately differentiated in 15 cases, and poorly differentiated in 30 cases). No patient received any radiotherapy or chemotherapy. The size of tumor was analyzed by maximum diameter. The patients were staged according to the international TNM system by International Union against Cancer (UICC).

Immunohistochemistry and evaluation criteria

Rabbit anti-human S100A4 polyclonal antibody and mouse anti-human VEGF monoclonal antibody were purchased from NeoMarkers. Two consecutive sections of each specimen were incubated. Immunohistochemistry staining was performed according to the manufacture's instructions. The tissue used as a negative control was incubated with PBS instead of primary antibody. The tissue known to highly express VEGF and S100A4 was used as positive control.

For each slide, cells positive for VEGF or S100A4 were counted and evaluated under 5-10 fields at 200 × magnification (cells counted: 100-200), and the percentage of positive cells was calculated. Cells were considered positively immune stained when nuclei and cytoplasm were stained. The distribution of stained S100A4 was evaluated with the percentage of stained cells scored as 0: < 5%, 1: 5%-25%, 2: 26%-50%, 3: 51%-75%, 4: > 75% and staining intensity scored as 1: buff, 2: buffy, 3: puce. When the multiplication of the two scores was greater than or equal to 2, S100A4 was considered positively stained. VEGF was considered positively stained when brown-stained granules were observed in cytoplasm and the percentage of positive cells was greater than 10%.

Statistical analysis

The correlation between S100A4/VEGF expression and clinicopathological parameters was evaluated by chi-square (χ^2) test or Fisher's exact test. Survival curves were plotted using the Kaplan-Meier method. Survival rates for different groups were compared using the log-rank test. Predictors for prognosis of the patients were assessed using Cox multiple hazards regression analysis. Statistical analysis was carried out using the SPSS 13.0. $P < 0.05$ was considered statistically significant.

RESULTS

Expression of S100A4 and VEGF

S100A4 was immunoreactive in cytoplasm and nuclei (Figure 1A). VEGF was immunoreactive mainly in cytoplasm (Figure 1B). Of the 62 pancreatic cancer patients, 38 (61.3%) had positive S100A4 expression and 24 (38.7%) negative S100A4 expression. Thirty of the 38 (78.9%)

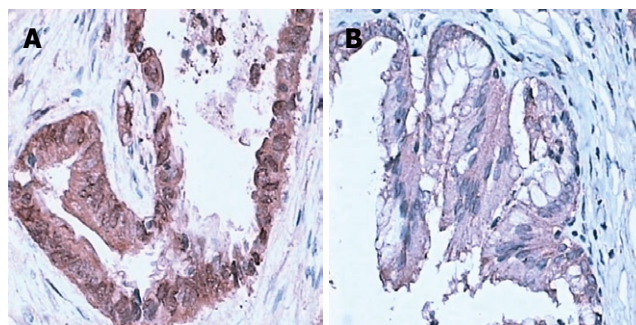


Figure 1 Positive expression of S100A4 (A) and VEGF (B) in pancreatic cancer ($\times 200$).

Table 1 Correlation analysis of S100A4 and VEGF expression in pancreatic cancer

S100A4	VEGF		P value
	(+)	(-)	
(+)	30	8	< 0.001
(-)	7	17	

Significance was estimated with χ^2 test.

patients with positive S100A4 expression had positive VEGF expression. Seventeen of the 24 (70.8%) S100A4-negative patients had negative VEGF expression. The positive correlation between expression of S100A4 and VEGF was statistically significant ($P < 0.0001$) (Table 1).

The correlation between S100A4/VEGF expression and clinicopathological parameters was analyzed (Tables 2-3). Tumors with their maximum diameter greater than 4 cm had a higher S100A4 expression than those with their maximum diameter less than 4 cm. Tumors at III + IV stage had a higher S100A4 expression than those at I + II stage. The correlation between S100A4 expression and tumor size and TNM stage was statistically significant. VEGF expression was not significantly related with the clinicopathological parameters.

Correlation between expression of S100A4 and VEGF and prognosis of patients

The 62 patients were followed up till December 2006 and their median survival time was 290.6 d. The 1-, 2-, and 3-year survival rate was 37%, 14%, and 7%, respectively. The median survival time of the S100A4 positive and negative patients was 232.8 d and 535.5 d, respectively, while the median survival time of the VEGF positive and negative patients was 229.7 d and 541.6 d, respectively. The survival curve was better for patients with S100A4-negative cancer than for those with S100A4-positive cancer ($P < 0.001$; log-rank test) (Figure 2A). The survival curve was better for patients with VEGF-negative cancer than for those with VEGF positive cancer ($P < 0.001$; log-rank test) (Figure 2B).

According to the expression of S100A4 and VEGF, pancreatic cancer patients were subdivided into four groups: (1) S100A4(+)/VEGF(+), (2) S100A4(+)/VEGF(-), (3) S100A4(-)/VEGF(+), (4) S100A4(-)/VEGF(-). Patients in the S100A4(-)/VEGF(-) group had a significantly

Table 2 Correlation between S100A4 expression and clinicopathological parameters in pancreatic cancer, *n* (%)

Clinicopathological parameters	Cases (<i>n</i>)	S100A4 expression		<i>P</i> value
		Positive	Negative	
Age (yr)				
≥ 70	24	15 (62.5)	9 (37.5)	> 0.05 ¹
< 70	38	23 (60.5)	15 (39.5)	
Gender				
Male	36	22 (61.9)	14 (38.1)	> 0.05 ¹
Female	26	16 (61.5)	10 (38.5)	
Differentiation				
Well	17	12 (70.6)	5 (29.4)	> 0.05 ¹
Moderately	15	9 (60.0)	6 (40.0)	
Poorly	30	17 (56.7)	13 (43.3)	
Tumor size (cm)				
< 2.0	3	0 (0)	3 (100)	< 0.05 ²
2.0-4.0	38	21 (55.3)	17 (44.7)	
> 4.0	21	17 (81.0)	4 (19.0)	
Lymph node metastasis				
(-)	13	5 (38.5)	8 (61.5)	> 0.05 ¹
(+)	49	33 (67.3)	16 (32.7)	
Distant metastasis				
(-)	39	23 (59.0)	16 (41.0)	> 0.05 ¹
(+)	23	15 (65.2)	8 (34.8)	
TNM stage				
I + II	30	14 (46.7)	16 (53.3)	< 0.05 ¹
III + IV	32	24 (75.0)	8 (25.0)	

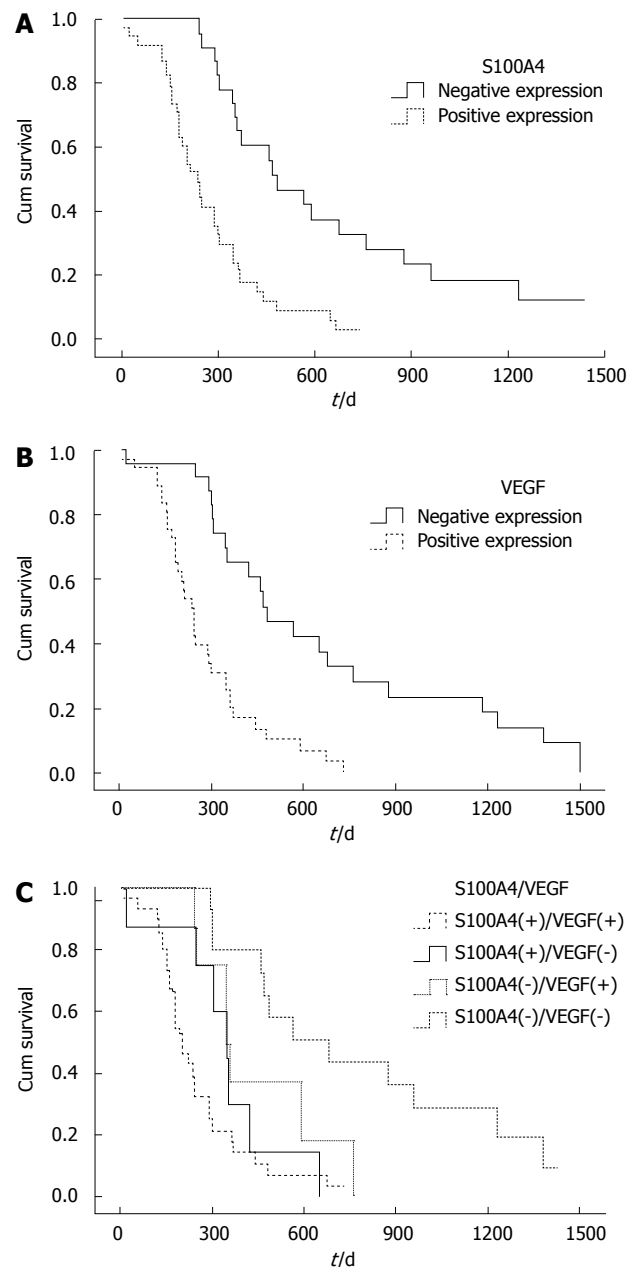
¹Significance was estimated with χ^2 test; ²Significance was estimated with Fisher's exact test.

Table 3 Correlation between VEGF expression and clinicopathological parameters in pancreatic cancer, *n* (%)

Clinicopathological parameters	Cases (<i>n</i>)	VEGF expression		<i>P</i> value
		Positive	Negative	
Age (yr)				
≥ 70	24	17 (70.8)	7 (29.2)	> 0.05
< 70	38	20 (52.6)	18 (47.4)	
Gender				
Male	36	24 (66.7)	12 (33.3)	> 0.05
Female	26	13 (50)	13 (50)	
Differentiation				
Well	17	10 (58.8)	7 (41.2)	> 0.05
Moderately	15	8 (53.3)	7 (46.7)	
Poorly	30	19 (63.3)	11 (36.7)	
Tumor size (cm)				
< 2.0	3	2 (66.7)	1 (33.3)	> 0.05
2.0-4.0	38	21 (55.3)	17 (44.7)	
> 4.0	21	14 (66.7)	7 (33.3)	
Lymph node metastasis				
(-)	13	7 (53.8)	6 (46.2)	> 0.05
(+)	49	30 (61.2)	19 (38.8)	
Distant metastasis				
(-)	39	24 (61.5)	15 (38.5)	> 0.05
(+)	23	13 (56.5)	10 (43.5)	
TNM stage				
I + II	30	17 (56.7)	13 (43.3)	> 0.05
III + IV	32	20 (62.5)	12 (37.5)	

Significance was estimated with χ^2 test.

better prognosis than those in the other three groups, and their median survival time was 678 d. Patients in the S100A4(+)/VEGF(-) group had a poorer prognosis than those in the S100A4(-)/VEGF(+) and S100A4(-)/VEGF(-) groups ($P < 0.05$; log-rank test). However, there was no

**Figure 2** Survival curves for group of pancreatic cancer patients according to S100A4 expression (A), group of pancreatic cancer patients according to VEGF expression (B), and four subgroups of pancreatic cancer patients according to the expression of S100A4 and VEGF (C).

significant difference between the S100A4(+)/VEGF(-) and S100A4(+)/VEGF(-) groups (Figure 2C).

The prognostic value of following parameters was analyzed, including age, differentiation of tumor, size of tumor, lymph node metastasis, distant metastasis, TNM stage and expression of S100A4 and VEGF. The influence of these parameters was evaluated by the Cox proportional hazards model. The results of these analyses showed that distant metastasis, expression of S100A4 and VEGF were significant independent prognostic predictors (Table 4).

DISCUSSION

In this study, the expression of S100A4 and VEGF was evaluated in relation to the clinicopathological parameters

Table 4 Cox multivariate regression analysis of clinicopathological features as a prognostic predictor

	B	SE	Wald	df	P value	OR (95% CI)
Distant metastasis	1.101	0.345	10.163	1	0.001	3.006 (1.528, 5.914)
S100A4	0.893	0.338	6.989	1	0.008	2.443 (1.260, 4.736)
VEGF	0.821	0.340	5.815	1	0.016	2.272 (1.166, 4.426)

of pancreatic cancer. Of the 62 pancreatic cancers patients, 61.3% were positive for S100A4 expression. Pancreatic cancer with a large size and high TNM stage had a higher S100A4 expression. Over-expression of S100A4 was significantly correlated with tumor size, TNM stage and a poor prognosis. These results suggest that over-expression of S100A4 might enhance cell motility and invasiveness.

It was reported that expression of S100A4 is significantly correlated with lymph node metastasis in several types of cancer, such as breast, colorectal, stomach, lung, and thyroid carcinoma^[19-23]. Our results show that positive S100A4 expression was higher in patients with lymph node metastasis than in patients without lymph node metastasis. However, there was no significant difference in S100A4 expression between the two groups, suggesting that expression of S100A4 is not closely related to lymph node metastasis. Further study is needed to confirm our findings.

S100A4 protein may modulate cell cycle, cell motility, invasiveness and adhesion. In cancer cells, S100A4 protein regulates protein kinase C phosphorylation of the heavy chain of nonmuscle myosin in a calcium-dependent manner, resulting in enhanced cell motility and invasiveness^[24,25]. Merzak *et al*^[26] reported that expression of S100A4 is closely correlated with *in vitro* invasiveness of glioma cells. Moreover, increased levels of S100A4 confer metastasis of non-metastatic epithelial cell line *in vivo*^[27,28]. Ambartsumian *et al*^[29] reported that S100A4 induces tumor progression by stimulating angiogenesis. All these findings demonstrate that S100A4 plays an important role in tumor growth, invasion, metastasis and angiogenesis.

VEGF is a mitogen and motor of vascular endothelial cells and also an important factor for angiogenesis. It can induce proliferation and migration of vascular endothelial cells, and formation of blood capillary lumens. VEGF increases vascular permeability and stimulates vascular endothelial cells to secrete proteinase and small molecules. In 1993, Brown *et al*^[30] firstly reported the high expression of VEGF in pancreatic cancer specimens, which is closely correlated with the growth and invasion of pancreatic cancer. In our study, S100A4 and VEGF expression in pancreatic cancer was detected, showing that expression of S100A4 and VEGF is closely correlated. The mechanism of S100A4 and VEGF expression and their relationship with the development and progression of pancreatic cancer deserve further study at molecular level.

In conclusion, expression of S100A4(-)/VEGF(-) cancer can be used as a marker to predict the survival of patients. Distant metastasis, positive S100A4 and VEG are highly independent prognostic predictors. Expression of S100A4 and VEGF can be used as an indicator of prognosis in patients with pancreatic cancer. Exploitation

and application of S100A4- or VEGF-targeted tumor inhibitors can decrease the recurrence or metastasis rate of pancreatic cancer and improve the prognosis of patients.

COMMENTS

Background

The prognosis of pancreatic cancer is very poor. Many patients are not diagnosed until at its advanced stage. Current treatment for the disease is surgery in combination with radiotherapy or chemotherapy. Growth and metastasis of pancreatic cancer were studied in order to improve its prevention and treatment.

Research frontiers

S100A4 protein expression in different kinds of cancer is closely correlated with the growth, invasion and metastasis of pancreatic cancer. The mechanism of S100A4 protein underlying the growth, invasion and metastasis of tumor is a hot topic in recent researches.

Innovations and breakthroughs

The expression of S100A4 and VEGF in pancreatic cancer and their correlation with prognosis of pancreatic cancer patients were studied. Our results show that expression of S100A4 and VEGF is positively correlated with pancreatic cancer.

Applications

By detecting tumor-associated proteins S100A4 and VEGF, we evaluated the biological characteristics and prognosis of pancreatic cancer, which can improve our understanding of pancreatic cancer and provide scientific basis for the application of S100A4 and VEGF inhibitors in treatment of pancreatic cancer.

Terminology

S100 protein: a member of the large family of soluble acid calcium-binding proteins, first discovered and designated by Moore in 1965. S100 protein can be completely dissolved in saturated ammonium sulfate, and the family consists of 21 members, like S100A1-A13, S100B, S100P, Profilaggrin, Trichohyalin and Reperin, etc. S100 protein regulates interaction between Ca⁺⁺ and target-protein, and is involved in a variety of physiological functions, such as cell proliferation and differentiation, cell cycle control, regulation of enzyme activity, and calcium-dependent transcriptional regulation.

Peer review

In this nice research, the authors investigated the expression of S100A4 and VEGF in pancreatic cancer and discussed the prognostic significance and clinical pathological features of S100A4 and VEGF. The research may offer certain contribution to the treatment of pancreatic cancer. The design of the study is scientific and the results are reliable and have clinical application values.

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RAPID COMMUNICATION

Double-balloon enteroscopy reliably directs surgical intervention for patients with small intestinal bleeding

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Lin MB, Yin L, Li JW, Hu WG, Qian QJ. Double-balloon enteroscopy reliably directs surgical intervention for patients with small intestinal bleeding. *World J Gastroenterol* 2008; 14(12): 1936-1940 Available from: URL: <http://www.wjgnet.com/1007-9327/14/1936.asp> DOI: <http://dx.doi.org/10.3748/wjg.14.1936>

Abstract

AIM: To evaluate preoperative double-balloon enteroscopy for determining bleeding lesions of small intestine, thus directing selective surgical intervention.

METHODS: We retrospectively reviewed 56 patients who underwent double-balloon enteroscopy to localize intestinal bleeding prior to surgical intervention, and compared enteroscopic findings with those of intraoperation to determine the accuracy of enteroscopy in identifying and localizing the sites of small intestinal bleeding.

RESULTS: Double-balloon enteroscopy was performed in all 56 patients in a 30-mo period. A possible site of blood loss was identified in 54 (96%) patients. Enteroscopy provided accurate localization of the bleeding in 53 (95%) of 56 patients, but failed to disclose the cause of bleeding in 4 (7%). There was one case with negative intraoperative finding (2%). Resection of the affected bowel was carried out except one patient who experienced rebleeding after operation. Gastrointestinal stromal tumor (GIST) was most frequently diagnosed (55%).

CONCLUSION: Double-balloon enteroscopy is a safe, reliable modality for determining bleeding lesion of small intestine. This technique can be used to direct selective surgical intervention.

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Key words: Double-balloon enteroscopy; Small intestine bleeding; Surgery

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INTRODUCTION

Small intestinal bleeding is uncommon, accounting for only 2%-10% of gastrointestinal bleeding cases^[1]. Localization of the bleeding site in small intestine is critical to planning appropriate therapy. But small intestine is roughly three meters in length and tortuously folded in the peritoneal cavity, which poses a challenge for endoscopies, computed tomography, scintigraphy and angiography to pinpoint the bleeding sources. Gastrointestinal hemorrhage is described as “obscure” if the bleeding site is not identified by these methods^[2]. Double-balloon enteroscopy was first introduced in 2001 as an important advance in the exploration of the small intestine^[3]. Compared with other methods, it has the advantage of direct visualization of the intestinal lumen, permitting biopsy^[4]. However, it is unclear whether enteroscopy alone is accurate enough to be used to direct appropriate surgical intervention. To evaluate preoperative double-balloon enteroscopy in determining bleeding focus of small intestine, we reviewed the results of enteroscopy in 56 patients who had small intestinal bleeding and underwent surgery.

MATERIALS AND METHODS

Patients and methods

We reviewed retrospectively the records of 56 consecutive patients who underwent surgery from June 2003 to December 2005 for small bowel bleeding in whom preoperative double-balloon enteroscopy was performed. The group of patients consisted of 26 females and 30 males with a mean age of 58 years (range from 14-75 years). The bleeding history ranged from 1 wk to 10 years. The main presenting features were melaena or maroon stool. The mean hemoglobin level was 65 g/L.

Table 1 Summary of diagnostic studies in 56 patients with small intestinal bleeding prior to enteroscopy and surgery

	No. of patients	No. of procedures
Barium enema	29	29
Gastroscopy	56	72
Colonoscopy	52	54
Angiography	9	9
CT	56	63
Tc ⁹⁹ m-labeled red blood cells scintigraphy	5	5
Capsule endoscopy	7	7

Table 2 Endoscopic and histological findings in patients undergoing enteroscopy for small bowel bleeding

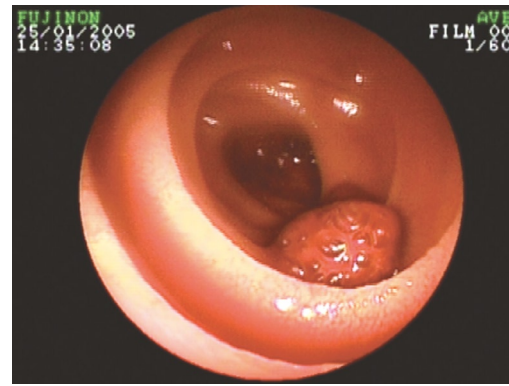
Endoscopic findings	No. of patients	Histology	No. of patients
Neoplasm	43	Stromal tumor	29
		Adenocarcinoma	7
		Adenoma	2
		Pheochromocytoma	1
		Lymphoma	1
		Hemangioendothelioma	1
		Leiomyosarcoma	1
		Inflammatory polyp	1
Vascular lesion	5	AVM	2
		Hemangiomas	1
		Stromal tumor	1
		Meckel's diverticulum	1
Meckel's diverticulum	3	Meckel's diverticulum	3
Crohn's disease	1	Ulcer	1
Peutz-Jeghers syndrome	1	Peutz-Jeghers syndrome	1
Polyp	1	Negative	1
Negative case	2	Stromal tumor	1
		Ulcer	1

(range from 35-98 g/L). The mean number of packed red blood cell unit transfused to each patient prior to surgery was 10 U. Coagulation parameters were normal in all patients. Each patient received an extensive diagnostic evaluation with 4 diagnostic procedures including esophagogastroduodenoscopy, colonoscopy, computed tomography, scintigraphy and barium enema, as well as angiography, and capsule endoscopy in some patients, but all failed to elucidate a possible bleeding site. The diagnostic workup for the patients prior to referral is summarized in Table 1. Two patients underwent surgery previously for bleeding.

Small bowel enteroscopy

Double-balloon enteroscopy was performed with a Fujinon EN450P5 video enteroscope (Fujinon, Saitama, Japan) with a working length of 2.0 m. EN450P5 is a thinner endoscope with an external diameter of 8.5 mm, and forceps channel diameter of 2.2 mm. The instrument is forward-viewing with a 120° angle of view.

The endoscopy can be used both from the mouth (anterograde approach) and anus (retrograde approach). It is highly possible that the entire small intestine can be observed by the endoscopy using the combination of both routes. The anterograde approach was used in 50

**Figure 1** Enteroscopy identified a polyp 1.5 cm in diameter in jejunum.

patients, the retrograde approach in 2 patients, and both in 4 patients.

RESULTS

Small bowel enteroscopy was performed in all 56 patients. A possible site of blood loss was identified in 54 (96%) patients. Neoplastic lesions were visualized in 43 patients, vascular malformation in 5, Meckel's diverticulum in 3, Peutz-Jeghers syndrome in 1, Crohn's disease in 1, polyp in 1 and negative finding in 2 patients.

All patients were surgically explored immediately after the enteroscopy. Thirty-six patients underwent laparoscopic surgery and 20 patients laparotomy. The final diagnoses of all patients are summarized in Table 2. GIST was the most frequent diagnosis in our study group (Figure 1). Thirty-one (55%) patients had GIST (19 located within 1.0 m distal to the ligament of Treitz). Other diagnoses included adenocarcinoma in 7 (13%) patients (Figure 2), Meckel's diverticulum in 4 (7%), vascular malformation in 3 (5%), adenoma in 2 (3%), ulceration in 2 (3%), Peutz-Jeghers syndrome in 1 (2%), lymphoma in 1 (2%), Crohn's disease in 1 (2%), leiomyosarcoma in 1 (2%), hemangioendothelioma in 1 (2%), inflammatory polyp in 1 (2%), pheochromocytoma in 1 (2%), and negative findings in 1 (2%) patient.

In 53 of 56 patients, enteroscopy provided accurate localization of bleeding, but it failed to disclose the causes of bleeding in 4 patients. Three patients were diagnosed with vascular malformation with preoperative enteroscopy, but the surgical specimen revealed Meckel's diverticulum, GIST and ulcer respectively. In two patients who had a normal small bowel at total enteroscopic examinations, ulcer was found in 1 patient and GIST in the other by surgery. One patient had a jejunal a polyp with enteroscopy, but no visualized lesion was found in surgery (Figure 3).

The mean follow-up period for the series was 29.6 mo. One patient had rebleeding during the follow-up period.

DISCUSSION

Vascular lesions are the most common cause of intestinal bleeding, accounting for 70%-80% in the Western world^[5], but in China are neoplasms, accounting for 22.2%-60.9%^[6]. Other causes include Meckel's diverticulum, ulcerative



Figure 2 Stromal tumor of small intestine. **A:** Preoperative enteroscopy; **B:** Surgical exploration; **C:** Photomicrograph of the lesion (HE, $\times 100$).

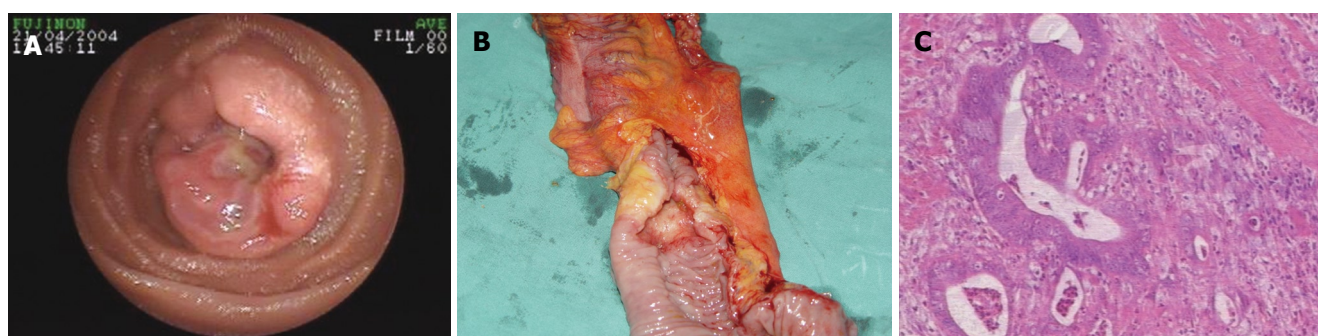


Figure 3 Adenocarcinoma of small intestine. **A:** Preoperative enteroscopy; **B:** Surgical exploration; **C:** Photomicrograph of the lesion (HE, $\times 100$).

disease, and Crohn's disease^[7]. Neoplasm was the most frequent diagnosis in our study group, accounting for 80%. The relative high rate was not reported previously. Small bowel bleeding always occur chronically and intermittently. Its intermittent and self-limiting nature may render correct diagnosis more difficult. In about 5% of patients, even multiple diagnostic modalities are unable to localize the source of bleeding^[8]. This causes a management problem as therapy depends on locating the site of blood loss. Double-balloon enteroscopy is an important advance in the exploration of the small bowel, especially for lesions discovered beyond the reach of standard endoscopy^[9]. The advantage of the technique over push enteroscopy is that extend portions of the small bowel can be viewed. It has been reported that push enteroscopy successfully identified the source of bleeding in 24%-75% of patients^[10-13]. Recently, Zhong reviewed 20 patients undergoing double-balloon enteroscopy for obscure gastrointestinal hemorrhage and reported a correct diagnosis in 80% cases^[14]. Although the diagnostic efficiency varies in different series, the indications and diagnostic yield of enteroscopy have yet to be fully defined. As shown by our experience, the indications for enteroscopy were classified into four groups: (1) Unexplained disease of small bowel such as digestive bleeding, abdominal pain and diarrhea. (2) Incomplete intestinal obstruction. (3) Diagnosis was made but the extension of small bowel lesion needs assessment. (4) Follow-up after treatment for small bowel disease. Of the 56 patients in this series, histopathologic examination confirmed the endoscopic findings in 52 (93%) cases. Though enteroscopy failed to disclose the causes of bleeding in 4 patients, surgery revealed that enteroscopy

provided the accurate localization of bleeding in 53 of 56 patients. Therefore, enteroscopy is a reliable modality for accurately localizing the site of small bowel bleeding. These enable surgeons confidently provide appropriate surgical intervention.

The small intestine represents approximately 75% of the total length of the gastrointestinal tract and more than 90% of the mucosal surface, but fewer than 2% of all gastrointestinal malignancies originate in the small bowel^[15]. It has been estimated that 35%-50% of the small bowel tumors are adenocarcinomas, 20%-40% are carcinoids and about 14% are lymphomas^[16,17]. GIST is uncommon mesenchymal tumors, accounting for approximately 0.1%-0.3% of all gastrointestinal neoplasms^[18]. However, in this group of patients, the most common histological type is GIST. Among all the 42 patients with small bowel tumors, 31 patients had GIST. The high proportion of GIST has not been found in other studies. About 32% of gastrointestinal GIST have been found in the small bowel^[19]. GIST comprises a spectrum of variable malignancies ranging from benign to aggressive forms^[20]. Histologically, the distinction between benign and malignant tumors is not evident. Two of the strongest pathologic predictors of malignant behavior are size and mitotic count^[21]. According to Shiu, GIST > 6 cm in diameter should be considered a potential malignance^[22]. GISTs usually arises from or between the muscularis propria and the muscularis mucosa of the bowel wall and extends primarily toward the serosa^[23]. But small bowel enteroscopy is limited in its mucosal visualization and can only detect the lump within the bowel lumen. Therefore, preoperative enteroscopy has little value in predicting



Figure 4 Diverticula was visualized as angioma by preoperative double-balloon enteroscopy. **A:** Preoperative enteroscopy, angioma between jejunum and ileum; **B:** Diverticula 80 cm proximal to ileocecum. **C:** Photomicrograph of the lesion (HE, $\times 100$).

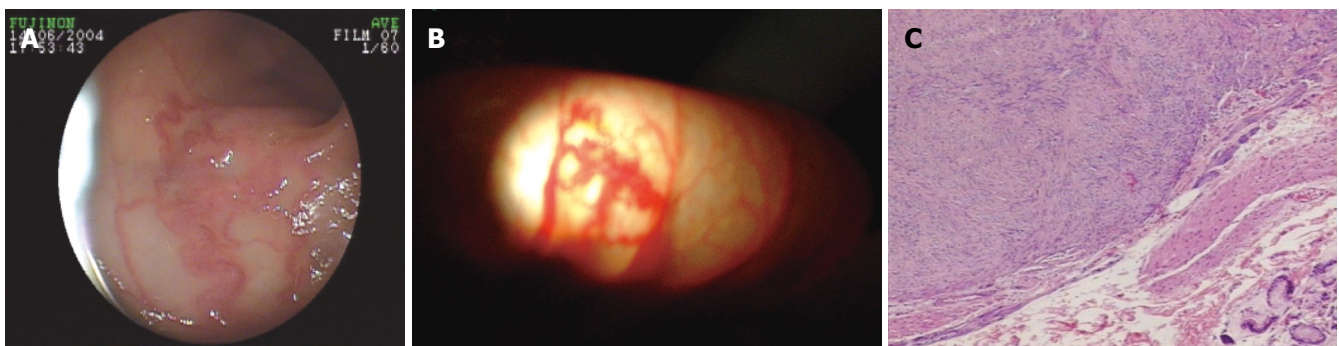


Figure 5 Vascular malformation of small intestine. **A:** Preoperative enteroscopy; **B:** Surgical exploration; **C:** Photomicrograph of the lesion (HE, $\times 100$).

benign or malignant behavior of GIST. However, to this kind of patients, enteroscopy can accurately localize the site of hemorrhage. The rate of rebleeding after surgery is very low. Interestingly, 19/31 stromal tumors arise within 1.0 m distal to the ligament of Treitz. Similar result was reported by Soderman^[24]. The main surgical complication in this area is prolonged gastroparesis and ileus. Four of 19 patients of our group had such complications.

Although most vascular lesions appear endoscopically similar, they consist of various pathological identities such as angiodysplasia (vascular ectasia), venous ectasia, telangiectasia, hemangiomas, arteriovenous malformation (AVM) and caliber -persistent artery (Dieulafoy's lesion)^[25]. Small bowel barium may not detect mucosal-based lesions such as vascular ectasias. ^{99m}Tc-labelled red blood cell scintigraphy and angiography are seldom useful in identifying the bleeding site in patients with chronic and moderate blood loss^[26]. Moreover, pooling of blood in the intestine may result in false localizations. One patient in this group had previously undergone a segmental resection based on angiographic findings. But reoperation revealed that the lesion identified by angiography is 2.0 m distal to the real bleeding lesion. At present, small bowel enteroscopy is not capable of marking the site of bleeding, so the areas of vascular malformation must be endoscopically reidentified by intraoperative enteroscopy so that the surgeon can precisely determine their location and the extent of surgical resection. Three cases of vascular lesions in our group underwent intraoperative enteroscopy and similar findings were described to that of preoperative enteroscopy (Figure 4).

Peutz-Jeghers syndrome (PJS) is an autosomal dominant inheritance characterized by hamartomatous gastrointestinal polyps and mucocutaneous pigmentation, and 70%-90% of patients have polyps in the small bowel^[27]. As the polyps are located diffusely throughout the small intestine, resection of the entire affected intestine may lead to short-bowel syndrome^[28]. The main advantage of enteroscopy is that surgeon can identify and resect the segment concentrating polyps. Endoscopic procedure allows effective snare of the residual polyps so as to avoid the risk of developing short-bowel syndrome^[29].

It is highly possible that entire small intestine can be observed by the double-balloon enteroscopy using the combination of both antegrade and retrograde routes^[30]. We agree with Pennazio M that identification of a single lesion is often enough for both diagnostic and therapeutic purposes^[31]. In our group, only 4 patients underwent both approaches and only one patient experienced rebleeding. Total small bowel enteroscopy identified a polyp 1.5 cm in diameter in this patient (Figure 3), but exploratory laparotomy with intraoperative endoscopy failed to find a source of blood loss. The reason may be that the manipulation of endoscopy can lead to the shedding of polyp, but the patient had rebleeding 3 mo after operation.

As the technology continues to evolve, the endoscopy will provide more valuable information. But the findings by enteroscopy has not been clarified, especially in the vascular lesion. For example, one patient in our group was diagnosed with hemangioma in the jejunum by preoperative enteroscopy (Figure 5A), the surgical specimen revealed Meckel's diverticulum (Figure 5B). In

conclusion, double-balloon enteroscopy is a safe, reliable modality in determining bleeding lesion of small intestine. As such, this technique can be reliably used to direct selective surgical intervention. Further studies should focus on analyzing and refining the clinical implications of endoscopic results.

COMMENTS

Background

Current techniques for detecting the source of bleeding in the small intestines have low diagnostic yields. The source of bleeding can be identified by colonoscopy, arteriography, scintigraphy, and barium radiology, but no bleeding sites are found in about 5%-10% of cases. Compared with other methods, double-balloon enteroscopy has the advantage of direct visualization of the intestinal lumen, permitting biopsy and treatment in some cases.

Research frontiers

Localization of the bleeding site in small intestine is critical to planning appropriate therapy.

Innovations and breakthroughs

In this retrospective study, the diagnostic yield of enteroscopy in identifying the source of bleeding was higher than the overall diagnostic yield of the conventional modalities. The results suggested that enteroscopy provided accurate localization of the bleeding in 53 (95%) of 56 patients.

Terminology

Gastrointestinal bleeding (OGIB) is generally defined as recurrent bouts of chronic bleeding for which no definite source has been discovered by routine investigations.

Applications

Double-balloon enteroscopy is a safe, reliable modality for determining bleeding lesion of small intestine. This technique can be used to direct selective surgical intervention.

Peer review

This is an interesting retrospective review of a significant number of cases. And the patients after double enteroscopy were subsequently subjected to surgery, and histological confirmation was done. The authors evaluated the use of double-balloon enteroscopy in identifying the source of small intestinal bleeding. Double-balloon enteroscopy can reliably direct surgical intervention for patients with small intestinal bleeding.

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Comparison of esomeprazole enteric-coated capsules vs esomeprazole magnesium in the treatment of active duodenal ulcer: A randomized, double-blind, controlled study

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Liang XY, Gao Q, Gong NP, Tang LP, Wang PL, Tao XH. Comparison of esomeprazole enteric-coated capsules vs esomeprazole magnesium in the treatment of active duodenal ulcer: A randomized, double-blind, controlled study. *World J Gastroenterol* 2008; 14(12): 1941-1945 Available from: URL: <http://www.wjgnet.com/1007-9327/14/1941.asp> DOI: <http://dx.doi.org/10.3748/wjg.14.1941>

Abstract

AIM: To evaluate the efficacy and tolerability of two different preparations of esomeprazole in healing duodenal ulcers.

METHODS: A total of 60 patients with active duodenal ulcers were enrolled and randomized to receive esomeprazole enteric-coated capsules (40 mg) or esomeprazole magnesium (40 mg), once daily, for 4 consecutive wk, with ulcer healing being monitored by endoscopy. Safety and tolerability were also assessed.

RESULTS: Fifty seven patients completed the whole trial. The ulcer healing rates at the end of wk 2 were 86.7% and 85.2% in the esomeprazole enteric-coated capsules and esomeprazole magnesium groups, respectively ($P = 0.8410$), and reached 100% at the end of wk 4 in both groups. Symptom relief at the end of wk 2 was 90.8% in the esomeprazole enteric-coated capsules group and 86.7% in the esomeprazole magnesium group ($P = 0.5406$); at the end of wk 4 symptom relief was 95.2% and 93.2%, respectively ($P = 0.5786$). Adverse events occurred in 16.7% of the esomeprazole enteric-coated capsules group and 14.8% of the esomeprazole magnesium group ($P = 1.0000$).

CONCLUSION: The efficacies of esomeprazole enteric-coated capsules and esomeprazole magnesium in healing duodenal ulcer lesions and relieving gastrointestinal symptoms are equivalent. The tolerability and safety of both drugs were comparable.

INTRODUCTION

Esomeprazole, the stereospecific S-isomer of omeprazole, is the first proton pump inhibitor (PPI) to be developed as a single isomer for use in the treatment of acid-related diseases^[1,2]. This optical isomer is subject to less first-pass metabolism and lower plasma clearance than omeprazole, thereby offering higher systemic bioavailability^[3,4]. Early studies have shown esomeprazole achieves greater and more sustained acid control than omeprazole, with a similar tolerability and safety profile^[5,6]. Furthermore, esomeprazole shows a more rapid onset of acid-suppression effect than omeprazole, and less inter-individual variation in acid control^[7,8]. Additionally, a recent crossover study demonstrated that esomeprazole at a standard dose of 40 mg once daily provides more effective control of gastric acid at steady state than standard doses of pantoprazole, lansoprazole and rabeprazole in patients with symptomatic gastroesophageal reflux disease (GERD)^[9,10]. In addition, esomeprazole treatment yields higher erosive esophagitis healing rates and provides sustained resolution of heartburn in more patients than any other currently available PPI^[11].

The current study investigated whether esomeprazole enteric-coated capsules (40 mg; synthesized by Chongqing Lummy Pharmacy, China) provides effective duodenal ulcer healing compared with esomeprazole magnesium (40 mg; Nexium, AstraZeneca Inc), when administered once daily for 4 wk, in a Chinese population.

MATERIALS AND METHODS

Patients

A randomized, double-blind, double-dummy, parallel-controlled study was conducted in accordance with the ethical principles of the Declaration of Helsinki and internationally accepted guidelines for clinical trials in patients with duodenal ulcer disease. Each protocol was approved by an independent ethics committee prior to study commencement. All patients provided written informed consent before entry into the study. The randomization scheme was computer generated. A centralized allocation method was used to assign patients to a treatment group.

Men and women aged 18-65 years, with no more than two active endoscopically confirmed duodenal ulcers (less than 2 cm in diameter), were recruited into the study from April 2006 to July 2006. Major exclusion criteria included: Pregnancy or lactation, any clinically significant abnormal laboratory values at entry, multiple drug allergies, prior gastric surgery, and concurrent treatment with corticosteroids or non-steroidal anti-inflammatory drugs. Discontinuation of any previous PPI therapy was required at least 7 d before randomization. No antisecretory drugs, including H₂-receptor antagonists, or any other agents known to alter the pharmacokinetics of PPI, were allowed during the study or within 1 wk before entry. In addition, patients were excluded from the study if they had esophageal erosions or ulceration, esophageal and/or gastric varices, gastric ulcer, pyloric stenosis, endoscopic evidence of active gastrointestinal bleeding or Zollinger-Ellison syndrome. Other exclusion criteria concerned concurrent renal or hepatic insufficiency, treatment for cancer and any history of drug or alcohol dependence.

Of the 60 patients enrolled in the study, 30 in the treatment group received esomeprazole enteric-coated capsules and 30 in the positive control group received esomeprazole magnesium, and 95% of patients completed the study. Three patients (5%) discontinued the study, all in the esomeprazole magnesium group, and were excluded from the evaluable cohort because of consent withdrawal. The baseline demographic and clinical characteristics of the 57 patients in the evaluable cohort, gender, age, height, blood pressure, heart rate, duration of disease, pre-entry score and initial ulcer size and number, were not different between the two groups (Table 1). Overall, the population was predominantly male (70.2%) and most patients were less than 55 years of age (80.7%). Approximately one-third of patients smoked and consumed alcohol, and this proportion was comparable between the two groups. Compliance with study medication was high during 4-wk treatment period, with more than 90% of the patients in each treatment group taking over 75% of the prescribed drugs.

Study procedures

Eligible patients were randomly assigned in a double-blind fashion to one of the two groups: The treatment group received esomeprazole enteric-coated capsules (40 mg; Chongqing Lummy Pharmacy, China) and an esomeprazole magnesium-matching placebo, and the other group received esomeprazole magnesium (40 mg; Nexium,

Table 1 Baseline demographic and clinical characteristics (% mean \pm SD)

Characteristics	Esomeprazole enteric-coated capsules (n = 30)	Esomeprazole magnesium (n = 27)	Statistics	P value
Gender, n (%)				
Male	20 (66.7)	20 (74.1)		
Female	10 (33.3)	7 (25.9)	$\chi^2 = 0.3725$	0.5416
Age (yr)	44.2 \pm 11.9	43.6 \pm 11.1	$t = -0.1981$	0.8437
Height (cm)	163.1 \pm 7.2	164.7 \pm 7.0	$t = 0.8339$	0.4079
Systolic BP (mmHg)	114.5 \pm 10.5	111.6 \pm 8.2	$t = -1.1730$	0.2459
Diastolic BP (mmHg)	73.4 \pm 7.4	74.3 \pm 9.2	$t = 0.3901$	0.6980
Heart rate (bpm)	73.8 \pm 8.5	75.1 \pm 11.3	$t = 0.4713$	0.6393
Duration of DU (mo)	58.3 \pm 61.2	58.6 \pm 53.7	$t = 0.0178$	0.9859
Total score of symptoms	5.5 \pm 2.6	5.2 \pm 1.6	$t = -0.5580$	0.5794
Ulcer diameter (mm)	7.7 \pm 2.6	7.5 \pm 2.4	$t = -0.2764$	0.7833
Ulcer number				
1, n (%)	23 (76.7)	23 (85.2)		
2, n (%)	7 (23.3)	4 (14.8)	$\chi^2 = 0.6621$	0.4158

SD: Standard deviation; BPM: Beats per minute; DU: Duodenal ulcer.

AstraZeneca Inc) and an esomeprazole enteric-coated capsule-matching placebo as a positive control group. The study began within 3 d of baseline endoscopy. Patients were administered the medicine once daily in the morning, 30 min before breakfast, for up to 4 wk. All medications were packaged and labeled identically to maintain blinding. Treatment allocation for each patient was provided in individually sealed and blinded randomization envelopes which were collected and checked by the monitor at the end of the study to ensure the integrity of the blinding.

Ulcer healing was determined by sequential endoscopies performed after 2 wk of therapy, and again after 4 wk if the ulcer was not healed. The primary efficacy variable was the rate of ulcer healing, defined as complete regeneration of the mucosa (re-epithelialization) with no visible mucosal breaks at the site of all ulcers identified during the study. An erosion at the original site of any ulceration was considered to be evidence of incomplete healing (Figure 1). Whenever possible, endoscopic examinations in individual patients were performed by the same endoscopist.

The secondary end-points of the frequency and intensity of epigastric pain, heartburn, regurgitation, flatulence, belching, nausea and vomiting were assessed at baseline and at the endoscopy visits. Gastrointestinal symptoms were graded on a four-point scale: 0 = none; 1 = mild (aware of symptoms, but easily tolerated); 2 = moderate (discomfort sufficient to cause interference with normal activities); and 3 = severe (incapacitating, with inability to perform normal activities). The patients recorded all of these items in diary cards on a daily basis. The investigator used the diary card information to complete the study case report forms and obtained the total score of all recorded symptoms. The relief of gastrointestinal symptoms was calculated as [(baseline total score-post-treatment total score)/(baseline total score)] \times 100%.

Assessment of adverse events

The safety and tolerability of the medication were assessed using physical examination at final visit, review

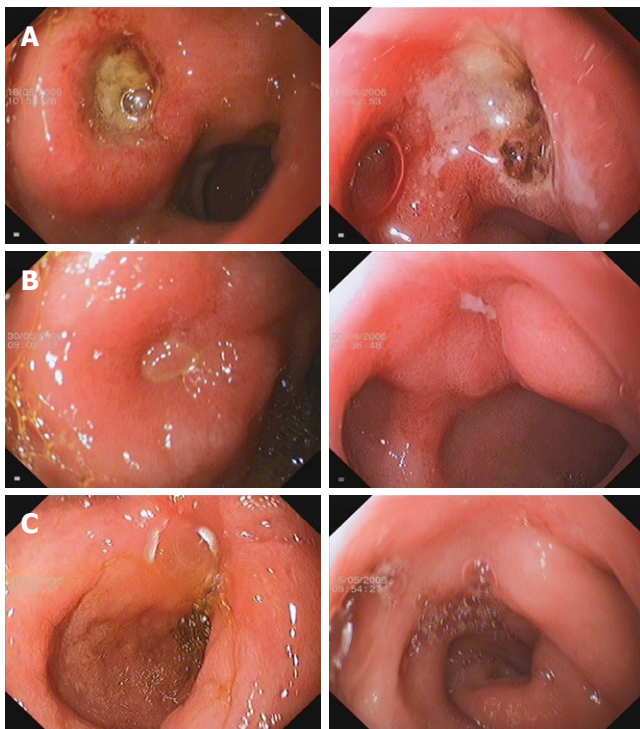


Figure 1 Comparison of Esomeprazole enteric-coated capsules treated group (left column) and Esomeprazole magnesium treated group (right column) in duodenal ulcer under endoscopy. **A:** Baseline duodenal ulcer under endoscopy; **B:** Duodenal ulcer under endoscopy at wk 2; **C:** Duodenal ulcer under endoscopy wk 4.

of adverse events as reported by patients at wk 4, and clinical laboratory evaluations at baseline and at the final visit. Clinical laboratory tests included serum chemistry, hematology and urine analysis. The causal relationship of an adverse event to the study drug was classified as being probable, possible or unlikely, and the intensity of the adverse event was rated as mild, moderate or severe. The action taken with study drug in response to the adverse event (none, treatment temporarily stopped, treatment discontinued) was also recorded.

Statistical analysis

Data were analyzed using SAS for Windows, version 6.12; the null hypothesis was rejected if P -values were ≤ 0.05 . The primary analysis was carried out on the per-protocol (PP) population, which included all randomized subjects who completed a full course of each treatment, had no appreciable loss of data, and had no major protocol violations. The significance of differences in categorical data was determined using the Pearson χ^2 or Monto-Carlo's exact test. The Student's t test and Mann-Whitney U test were used when appropriate. Results are reported as means and standard deviations.

RESULTS

Ulcer healing

The duodenal ulcer healing rates at wk 2 and 4 were compared between the two treatment groups (Figure 1). At wk 2, the healing rate was 86.7% in the esomeprazole enteric-coated capsules group compared with 85.2% in the esomeprazole magnesium group ($P = 0.8410$). At wk

Table 2 Adverse events during 4-wk therapy

Adverse event	Esomeprazole enteric-coated capsules ($n = 30$)	Esomeprazole magnesium ($n = 27$)
Dizziness	2	2
Diarrhea	1	0
Constipation	0	1
Face puffiness	1	0
Other	1 (cough)	1 (palpitation)
Total	5	4

4, 100% ulcer healing was documented in all patients. As shown in Figure 1, case 223 received esomeprazole enteric-coated capsules and case 181 received esomeprazole magnesium. Both patients had much improved duodenal ulcers at wk 2 and had complete ulcer healing at wk 4.

Relief of gastrointestinal symptoms

Assessment of gastrointestinal symptoms showed significant improvements in the frequency and intensity of epigastric pain, heartburn, regurgitation, flatulence, belching, nausea and vomiting at wks 2 and 4, and the two groups demonstrated comparable efficacy. At wk 2, the rate of symptom relief was 90.8% in the esomeprazole enteric-coated capsules treatment group compared with 86.7% in the esomeprazole magnesium positive control group ($P = 0.5406$). At wk 4, the rates of symptom relief in the two groups were 95.2% and 93.2%, respectively ($P = 0.5786$).

Safety and tolerability

Of the 60 patients with duodenal ulcers who were randomized with respect to medication, 57 patients received either esomeprazole enteric-coated capsules ($n = 30$) or esomeprazole magnesium ($n = 27$) for four wk, and were included in the safety and tolerability analysis. Only a few adverse events were documented with the following distribution: 5/30 patients (16.7%) in the esomeprazole enteric-coated capsules treatment group, and 4/27 patients (14.8%) in the esomeprazole magnesium positive control group (Table 2). There was no difference between the two groups ($P = 1.0000$). The reported adverse events during the trial were minor and did not require treatment interruption. There were no clinically relevant changes in blood pressure, heart rate or laboratory values during the study.

Of the 3 patients who withdrew from this study, one patient moved to another city because of a change in work place, and the other two patients rejected the gastroscopic operation because of complete symptom relief.

DISCUSSION

There are stereoselective differences in the metabolism of PPIs by the cytochrome P450 (CYP) isoenzymes 2C19 and 3A4, and this is the basis of the observed pharmacodynamic and clinical efficacy differences between esomeprazole and omeprazole^[12-15]. A study in which these enzymes were expressed from cDNAs suggested that CYP2C19 is responsible for 40% and 87% of the total intrinsic clearance of S- and R-omeprazole, respectively^[16],

indicating esomeprazole would be cleared more slowly *in vivo*^[16,17]. Several pharmacological studies using intragastric pH monitoring conducted in either healthy subjects or GERD patients have consistently established the superiority of standard dose esomeprazole over all other currently available standard PPI regimens^[18-22]. Recently, another S-isomer of pantoprazole has been used to investigate the efficacy in the treatment of acid-related disease; it has shown better efficacy in the control of GERD symptoms than its racemic mixture of pantoprazole^[23].

Miner *et al*^[9,10] demonstrated that, in a five-way crossover study, oral esomeprazole (40 mg) increased intragastric pH more rapidly and maintained intragastric pH above 4.0 longer than lansoprazole (30 mg), omeprazole (20 mg), pantoprazole (40 mg) and rabeprazole (20 mg) did in 34 *H. pylori*-negative patients with symptoms of gastroesophageal reflux disease. In addition, a recent study showed that esomeprazole (20 mg) was more effective at maintaining gastric pH above 4 for longer than lansoprazole (15 mg), pantoprazole (20 mg) and rabeprazole (10 mg)^[24].

Two randomized multicenter trials^[25,26] which used esomeprazole to treat DUs demonstrated that in *H. pylori*-positive patients with duodenal ulcer, 1 wk of esomeprazole (20 mg twice daily) triple therapy followed by placebo for 3 wk provides the same effective ulcer healing, *H. pylori* eradication and symptom control when compared with 1 wk of omeprazole (20 mg twice daily) triple therapy followed by a 3-wk period of omeprazole monotherapy (20 mg once daily). The authors concluded that 1 wk of esomeprazole-based triple therapy is sufficient to ensure high rates of ulcer healing without the need for follow-on PPI monotherapy in patients with uncomplicated duodenal ulcer disease. Besides, in GERD patients, esomeprazole demonstrated significantly higher healing rates at 4 and 8 wk than other standard dose PPIs, and the magnitude of the benefit that esomeprazole offers increases with the severity of the underlying reflux esophagitis^[11].

The present study investigated the efficacy and safety of esomeprazole enteric-coated capsules (synthesized by Chongqing Lummy Pharmacy, China) in the treatment of active duodenal ulcer disease, with esomeprazole magnesium (Nexium, AstraZeneca Inc)-treated patients used as a positive control group. Patients with active duodenal ulcers received esomeprazole (40 mg) once daily for four wk in both groups. At the end of second wk, duodenal ulcer healing was 86.7% in the treatment group and 85.2% in the positive control group ($P = 0.8410$), and at the end of fourth wk duodenal ulcer healing was 100% in both groups (Figure 1). In the improvement of gastrointestinal symptoms caused by active duodenal ulcer, the esomeprazole enteric-coated capsules treatment group showed 90.8% relief, which was greater than that of the esomeprazole magnesium positive control group (86.7%, $P = 0.5406$) at the end of the second wk; at the end of the fourth wk, 95.2% and 93.2% symptom relief was seen in the esomeprazole enteric-coated capsules treatment group and the esomeprazole magnesium positive control group, respectively ($P = 0.5786$).

Since the first PPI, omeprazole, was launched on the market in 1988, it has been widely used to treat acid-

related disorders and has demonstrated good efficacy and safety. Treatment with omeprazole over the mean period of 6.5 years causes histologic changes in the stomach which can be detected only by biopsy, but with no specific symptoms^[27]. Meanwhile, esomeprazole, the S-isomer of omeprazole, which had increased plasma concentrations and better clinical efficacy than omeprazole, should not be associated with any increase in unwanted effects. In studies involving large numbers of patients, the adverse event rates of esomeprazole have been proven to be similar to those recorded for omeprazole and placebo^[28,29]. Our data showed the adverse event rates were 16.7% in the esomeprazole enteric-coated capsules treatment group and 14.8% in the positive control group ($P = 1.0000$). The results of the current study are consistent with the findings of Richter *et al*^[30], who showed that in the 8-wk treatment of reflux esophagitis, the adverse event rates were 15.3% in the esomeprazole group and 15.1% in the omeprazole group. However, the adverse event rate was much lower in the study by Maton *et al*^[28]. In that study the adverse event rate of esomeprazole was about 3% compared with placebo, in 12-mo treatment for reflux esophagitis. The difference between these studies could be due to variations in therapy duration. Furthermore, in the current study, 3 patients in the esomeprazole magnesium group withdrew from this study, but none of these discontinuations were related to adverse events.

In conclusion, the results of this study indicate treatment with esomeprazole enteric-coated capsules (40 mg) is equivalent to treatment with esomeprazole magnesium (40 mg) in the healing of active duodenal ulcers and improving gastrointestinal symptoms, and that these treatments have similar safety and tolerability profiles.

COMMENTS

Background

Proton pump inhibitors (PPI) were introduced in the late 1980s and have emerged as the drug class of choice for the treatment of most acid-related disorders. Omeprazole was the first PPI on the market, followed by lansoprazole, rabeprazole, pantoprazole and esomeprazole, in that order. Conversely from the other available PPIs, which are all racemic mixtures, esomeprazole, the S-isomer of omeprazole, is the first PPI to be developed as a single isomer and demonstrates pharmacological and clinical benefits beyond those seen with racemic PPIs.

Research frontiers

Studies have shown esomeprazole at a standard dose of 40 mg once daily achieves greater and more sustained acid control than standard doses of any other currently available PPI, with good safety.

Innovations and breakthroughs

This study was designed to evaluate the efficacy and tolerability of two different preparations of esomeprazole, esomeprazole enteric-coated capsules and esomeprazole magnesium, in the healing of active duodenal ulcers.

Applications

This study indicates treatment with esomeprazole enteric-coated capsules is equivalent to treatment with esomeprazole magnesium in healing duodenal ulcer lesions and relieving gastrointestinal symptoms, and could be an alternative in the treatment of acid-related diseases.

Terminology

Isomers are compounds which have the same molecular formula but different

chemical structures. Depending on the types of differences there are between the structures, it is possible to classify isomers into various sub-types. Stereo-isomers contain the same functional groups and differ only in the arrangement of atoms in space.

Peer review

The study is performed well and carefully written.

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CASE REPORT

Investigation of the excluded stomach after Roux-en-Y gastric bypass: The role of percutaneous endoscopy

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Abstract

Accessing the bypassed portion of the stomach via conventional endoscopy is difficult following Roux-en-Y gastric bypass surgery. However, endoscopic examination of the stomach and small bowel is possible through percutaneous access into the bypassed stomach (BS) with a combined radiologic and endoscopic technique. We present a case of obscure overt gastrointestinal (GI) bleeding where the source of bleeding was thought to be from the BS. After conventional endoscopic methods failed to examine the BS, percutaneous endoscopy (PE) was used as an alternative to surgical exploration.

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Key words: Percutaneous endoscopy; Roux-en-Y gastric bypass; Gastrointestinal bleeding; Obesity; Bypassed stomach

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Gill KRS, McKinney JM, Stark ME, Bouras EP. Investigation of the excluded stomach after Roux-en-Y gastric bypass: The role of percutaneous endoscopy. *World J Gastroenterol* 2008; 14(12): 1946-1948 Available from: URL: <http://www.wjgnet.com/1007-9327/14/1946.asp> DOI: <http://dx.doi.org/10.3748/wjg.14.1946>

INTRODUCTION

Bleeding from the bypassed stomach (BS) in patients following Roux-en-Y gastric bypass surgery (GBS) is uncommon and can be a challenging diagnostic and therapeutic task. When the BS is not reachable by conventional ap-

proaches or more novel methods (e.g. double balloon endoscopy), surgical exploration may provide the only access to the BS and small bowel. Percutaneous endoscopy (PE) provides an opportunity to examine the BS and bowel and could be considered a practical alternative to surgical exploration.

CASE REPORT

Patient

A 66-year-old man status post Roux-en-Y GBS for morbid obesity 6 years prior presented to an outside institution with melena and hemodynamic instability. His hemoglobin (Hgb) was 10 gm, a drop from baseline Hgb of 15 gm. The patient was taking aspirin 81 mg daily. Upper endoscopy revealed expected post-surgical changes, and colonoscopy showed diverticulosis with no bleeding. A tagged RBC scan revealed a focus of activity in the right upper quadrant. Aspirin medication was stopped and the patient was discharged following cessation of clinical bleeding and a stable Hgb. He was readmitted 5 d later with recurrent melena and an Hgb of 8.5 gm. Repeat bleeding scan was negative, but CE revealed bleeding at 4 h into the recording (suspected jejunum). The Roux-en-Y anastomosis was not identified.

The patient was transferred to our institution for DBE, but the Roux-en-Y anastomosis was not reached, and no proximal source of bleeding was identified. Based on the suspicion that bleeding was coming from the BS, diagnostic and therapeutic alternatives including open or laparoscopic surgical exploration with gastroscopy and possible gastrectomy were considered. Surgery was considered high risk because of patient's obesity and other comorbid conditions. As an alternative to surgical exploration, we endeavored to examine the BS using a combined approach with interventional radiology and endoscopy.

Technique

After a thorough discussion of potential risks and benefits, informed consent was obtained. One milligram of intravenous glucagon was administered to prevent gastric peristalsis. Once the BS was identified with real-time trans-abdominal ultrasonography, a 19-gauge needle was passed into the collapsed lumen of the BS under sterile conditions. The stomach was then inflated with air while progress was monitored with fluoroscopy (Figure 1A).

Three gastric anchors were inserted into the central portion of the BS, pulling the gastric wall to the abdominal

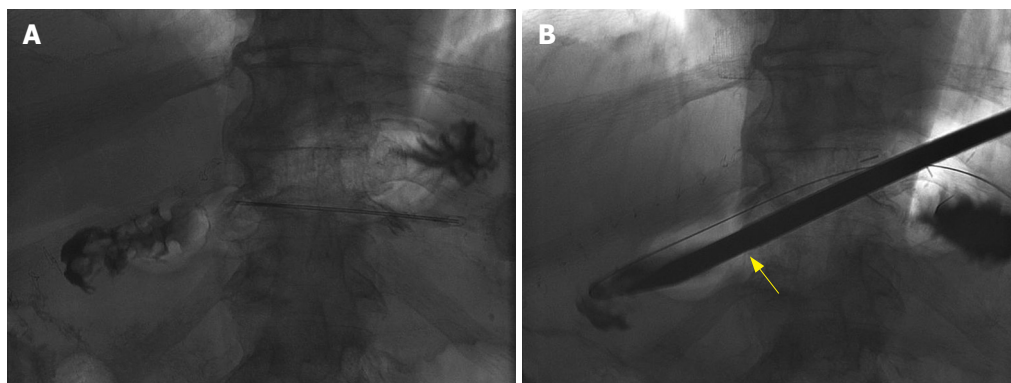


Figure 1 Fluoroscopic images of percutaneous endoscopy. **A:** Inflated bypassed stomach with air; **B:** Trochar (arrow) used to dilate the percutaneous track.

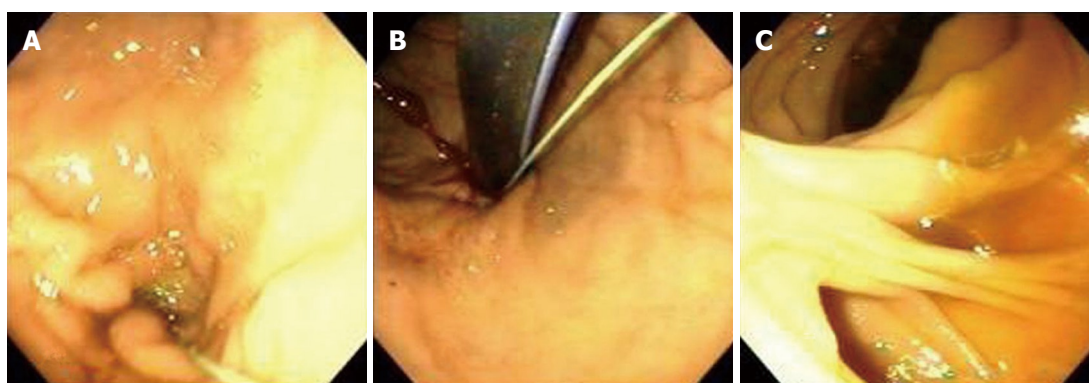


Figure 2 Endoscopic images by percutaneous endoscopy. **A:** Antegrade views of bypassed stomach; **B:** Retrograde views of bypassed stomach; **C:** Roux-en-Y anastomosis.

wall. A trochar was inserted, the tract was dilated, and a 30 French sheath was placed under fluoroscopic guidance (Figure 1B).

Through the sheath, a standard Olympus endoscope was introduced into the BS and examination was performed in antegrade and retrograde fashion (Figure 2A and B). The endoscope was advanced through the stomach and small bowel to the Roux-en-Y (Figure 3) anastomosis, where the two additional limbs of small bowel were examined for a distance of approximately 10 cm (Figure 2C). No source of bleeding was identified. After completion of the endoscopy, a 22 French gastrostomy tube (PEG) was placed in the BS for future access in the event of rebleeding. The patient experienced no clinical bleeding and had a stable Hgb, so he was discharged with the PEG (later changed to a button PEG) in place. As no bleeding occurred after 3 mo, the gastrostomy tube was removed.

DISCUSSION

The prevalence of obesity in the United States is reaching epidemic proportions. An estimated 30% of individuals met the criteria for obesity in 1999-2002^[1,2]. The increasing numbers of obese individuals have led to intensified interest in GBS. The estimated numbers of bariatric surgical procedures in United States have increased from 13 365 in 1998 to 72 177 in 2002^[3]. GBS is an effective and safe procedure; however, gastrointestinal (GI) bleeding after GBS can occur with an incidence of 0.8% to 4.4%^[4,5].

Bleeding after GBS has been classified as early (< 48 h) and late (> 48 h)^[6]. Late bleeding usually indicates luminal bleeding, whereas early bleeding may apply to bleeding intraluminally or into the abdominal cavity. The most com-

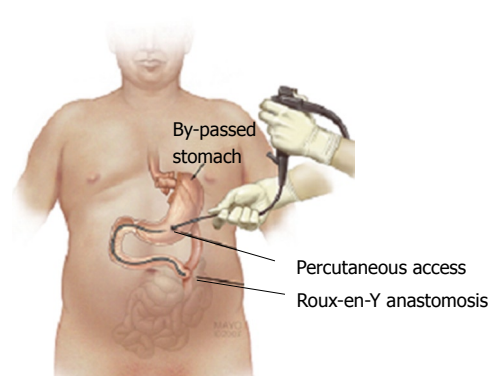


Figure 3 Illustration of the percutaneous endoscopy.

mon etiology of bleeding from BS is peptic ulcer disease (PUD), but the true incidence of bleeding from the BS is not known. One series reported only 8 of 3000 (0.26%) patients experienced GI bleeding from PUD involving the BS^[7].

Endoscopic examination of the BS is a challenge in this patient population as it is difficult to reach the BS with conventional endoscopy. Flickinger and colleagues described the use of a pediatric colonoscope in 1985. In that series of 78 procedures, 68% of the attempts to pass through the jejunostomy for retrograde evaluation of duodenum and BS were successful^[7]. Later, Sinar *et al* evaluated the BS by retrograde endoscopy and reported a successful procedure in 65% (33/51) of their patients^[8].

Percutaneous examination of the BS with contrast was first described by McNeely *et al* in 1987^[9]. In a series of 14 patients (11 nausea/pain, 3 GI bleeding), the radiocontrast

examination was successful in 13/14 patients. However, the specificity of the findings was low as only 20% (1/5) of the patients had confirmation of the suspected diagnosis. Endoscopic examination through percutaneous access with a bronchoscope was first reported in 1998^[10]. Since then, only a few case reports have been published utilizing PE to evaluate suspected pathology in the BS^[11,12].

New noninvasive techniques like virtual endoscopy have been evaluated for examination of BS in a small series^[13]. However, more studies are needed to define the utility of the technique. Recently a case series utilizing DBE reported successful examination of the BS in 83.3% (5/6) of the cases^[14]. The DBE technique enjoys several advantages over the more invasive percutaneous approach, but examination with DBE may be limited by difficult access, as with our patient, necessitating alternative approaches.

In this case no identifiable source of bleeding was found and hence the gastrostomy tube was left in place for future access. This is an advantage of the percutaneous approach as the stomach can be flushed and assessed in the event of bleeding. The access also allows a portal for endoscopy (requiring standard equipment and no special training). Fortunately, our patient had no further bleeding, so the gastrostomy tube was removed after 3 mo of observation.

In conclusion, this case highlights the value of PE to examine the BS after Roux-en-Y GBS. Advantages of this technique include direct access to the BS and the ability to leave an access point in the event of recurrent bleeding. Moreover, less endoscopic expertise is required compared to DBE, and the procedure is less invasive than surgery particularly in patients with multiple comorbidities.

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Conservative management of perforated duodenal diverticulum: A case report and review of the literature

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Abstract

Duodenal diverticula are a relatively common condition. They are asymptomatic, unless they become complicated, with perforation being the rarest but most severe complication. Surgical treatment is the most frequently performed approach. We report the case of a patient with a perforated duodenal diverticulum, which was diagnosed early and treated conservatively with antibiotics and percutaneous drainage of secondary retroperitoneal abscesses. We suggest this method could be an acceptable option for the management of similar cases, provided that the patient is in good general condition and without septic signs.

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Key words: Duodenal diverticula; Perforation; Conservative management

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INTRODUCTION

Duodenal diverticula are a relatively common condition.

Usually asymptomatic, they may become clinically evident when complicated, for example, with inflammation, bleeding or perforation. Perforation is the rarest complication and carries a high mortality. As this is exceptional, there are no well defined management recommendations. Below, we describe one approach to conservative management.

CASE REPORT

An 85-year-old female, with arterial hypertension as the only antecedent, arrived at the Emergency Department with an intense thoracic pain radiating to the upper abdomen, accompanied by cold sweating, which occurred suddenly while she was out walking. A few minutes later, she experienced nausea and vomiting. General exploration demonstrated arterial hypotension and epigastric pain on palpation. Blood analysis only showed leukocytosis ($15\,000/\text{mm}^3$ with 82% neutrophils). Thoracic and abdominal radiographs were normal. Due to a suspicion of dissecting aorta aneurism, a thoraco-abdominal computed tomography (CT) was performed, revealing perihepatic-free liquid, pneumoperitoneum and retroperitoneum next to the cava vein, secondary to perforation of a hollow viscus, probably of the duodenum (Figure 1).

In the initial hours following the patient's admission to hospital she improved with only intravenous fluid resuscitation, presenting with remittent fever and abdominal pain. It was decided to maintain conservative management with nasogastric decompression and intravenous Meropenem, which was then administered.

The nasogastric tube was retrieved on the fourth day, but she remained nil-by-mouth and continued to receive intravenous antibiotherapy. She had an uneventful evolution; on the sixth day, a Gastrografin swallow examination revealed a duodenal diverticulum in the second portion, without leakage of contrast at this time (Figure 2). A new abdominal CT showed an abscess surrounding the duodenal diverticula and another one, which was retrohepatic and subdiaphragmatic, containing air. Both were drained percutaneously. The patient was discharged on the twentieth day. Six months later she is asymptomatic.

DISCUSSION

The incidence of duodenal diverticula is estimated to be 5%-22% in a healthy population^[1,2]. Most of these are located along the pancreatic or mesenteric border,

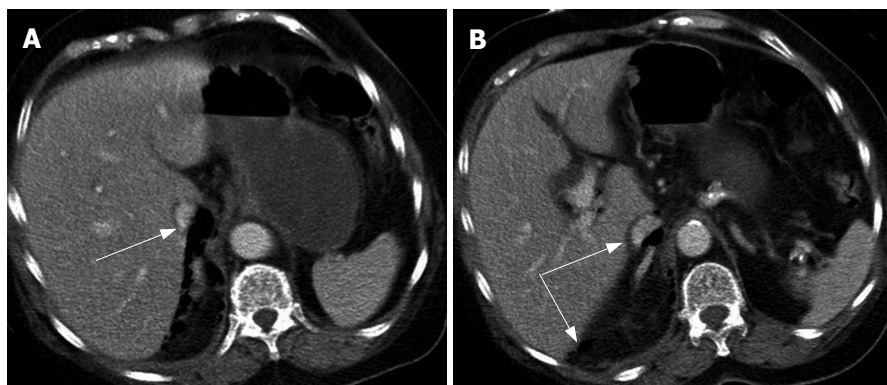


Figure 1 Abdominal CT showing perihepatic free liquid, pneumoperitoneum and retropneumoperitoneum next to the cava vein.

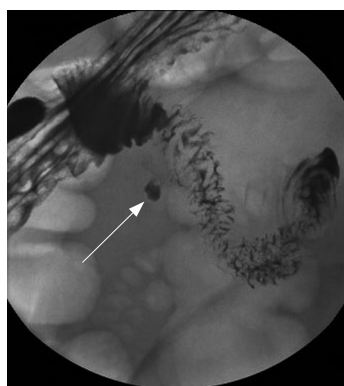


Figure 2 Gastrografin swallow examination revealing a duodenal diverticulum in the second portion without leakage of contrast.

mainly in the second part of the duodenum, and are really pseudodiverticula, as they do not involve all of the intestinal layers^[2-4]. Many of them are near the ampulla of Vater, and these are known as perivaterian or periampullary diverticula^[4].

They are asymptomatic until they develop complications. Many possible complications have been reported. The most frequent are inflammation, haemorrhage, pancreatitis and common bile duct obstruction. Perforation is considered to be the rarest complication, and is also the most serious, with a mortality of up to 20%^[5,6]. Only about 100 cases have been reported over the past two decades^[2].

Diagnosis is difficult, because the clinical presentation is non-specific, without pathognomonic signs or symptoms, and requires imaging. However, the most frequent presentation is acute pain in the right upper abdomen or epigastrium, associated with nausea and vomiting.

As the perforation is usually open to the retroperitoneum, it is possible there will be no signs of peritonism. Diverticulum perforation usually leads to retroperitoneal abscess formation and sepsis. It may also lead to the development of duodenocolic fistula with steatorrhea or gastrointestinal bleeding if perforation gets into the aorta^[6]. It is important to make a differential diagnosis with other right upper abdomen pathologies such as cholecystitis, cholangitis, pyelonephritis, perforated duodenal ulcers or even bottom right pneumonia.

In most instances plain abdominal radiographs or ultrasounds are used as the first imaging techniques with subtle findings, and preoperative diagnosis is usually incorrect^[2]. CT is the modality of choice^[7], usually demonstrating a thickened bowel wall, mesenteric fat inflammation

and an extraluminal collection of air and fluid, often retroperitoneal^[7-9]. It is frequently possible to identify the diverticulum itself.

The most common approach is surgical, although there exist only a few reports of conservative management with antibiotics and percutaneous drainage^[8,10-13]. Surgical intervention will depend on the clinical situation and intraoperative findings. If inflammation permits, the treatment of choice is, after Kocherizing the duodenum, diverticulectomy with single or double-layer duodenal closure. It is important to place drainage tubes, especially in the retroperitoneum if affected^[2,4,7]. A tongue of the greater omentum can be patched over the closure. Injury to the pancreatic or distal common bile duct can be avoided by placing a tube into the ampulla of Vater before dissecting the diverticulum.

When there is substantial inflammation of the duodenum, a diversion should be performed by a subtotal gastrectomy followed by Billroth II reconstruction, or a Roux-en-Y gastroenteroanastomosis. Only patients who are mildly affected are likely to benefit from non-operative management. In these patients, the perforation has probably already sealed spontaneously, or will do so within a short period of time.

Non-operative management consists of nasogastric decompression and wide spectrum antibiotics. It may be advisable to perform radiographic studies with water-soluble contrast between the fifth and seventh day, before starting oral alimentation with fluids. Formation of abscesses is likely, so percutaneous drainage must be easily accessible if conservative management is implemented. If a patient's condition worsens, conservative management must be abandoned in favour of surgery.

In conclusion, duodenal diverticulum perforation is a very rare but serious complication with a difficult diagnosis, normally requiring a CT. Very few cases which have been treated conservatively, for example, using antibiotics and posterior percutaneous drainage, have been reported. This option must only be tried in patients who are in a generally good condition with no septic signs. This must of course be replaced by surgery if the patient deteriorates.

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CASE REPORT

Paraneoplastic hyperinsulinism and secondary hypoglycaemia in a patient with advanced colon cancer: A rare association

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Abstract

We review the case of a 74-year-old patient with advanced colon cancer who suffered recurrent bouts of hypoglycemia. A state of inappropriate, non-suppressed hyperinsulinism in the presence of severe hypoglycemia was diagnosed. We finally discuss the known mechanisms behind fasting hypoglycemia in patients with advanced cancer, the diagnosis, and possible treatments of this rare paraneoplastic endocrine complication.

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Key words: Hypoglycemia; Colon cancer; Paraneoplastic; Hyperinsulinism; Tumour markers

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INTRODUCTION

Non-islet cell tumour hypoglycemia (NICTH) is a rare association between spontaneous hypoglycemia and tumours derived from tissues other than the pancreatic

islets^[1]. It was initially associated with abdominal soft tissue sarcomas, although other tumour types have been described. The most consistent finding is the overproduction of insulin or especially insulin-like growth factors by the tumour. We review the case of a patient with a sigmoid carcinoma with ectopic production of insulin and secondary hypoglycemia and the clinical diagnosis and management of this rare condition.

CASE REPORT

A 74-year-old male patient was diagnosed in September 2004 with a sigmoid cancer with advanced hepatic and pulmonary metastases. He had no previous medical history of interest. The primary tumour was surgically removed. The pathologic study showed a moderately differentiated adenocarcinoma; the TNM staging was pT4 N2 (12/15) M1, stage IV. After surgery, his performance status (PS) was 1. There were no baseline laboratory abnormalities. His CEA and CA 19.9 levels were 270.9 ng/mL and 1257.3 UI/mL, respectively.

Between November 2004 and April 2005, he received 1st line chemotherapy with 12 fortnightly cycles of infusional 5-fluorouracil and oxaliplatin (FOLFOX regimen). A radiologically stable disease was achieved after the 6th and 12th infusion, with a lowering of the CEA and CA19.9 levels. Overall tolerance to chemotherapy was good; however, treatment was finally stopped due to persistent grade 2 chronic neurotoxicity (CTC version 3) secondary to oxaliplatin. At that moment, he began follow-ups in our clinic.

In July 2005, the patient suffered a loss of consciousness in the early hours of the morning. The capillary blood glucose at that moment was low (45 mg/dL) and he was brought to the Emergency Room (ER). There were no other basic laboratory abnormalities. The electrocardiogram was normal, as was a CT brain scan. With further questioning, the patient referred in the last weeks similar episodes of poor sleep, frequent nightmares, dizziness and blurred vision, although with no loss of consciousness, in the early hours of the morning, which improved with the ingestion of food. The patient had also gained weight in the last weeks, as he had a craving for sweet foods; he also had learnt to avoid these episodes of dizziness during the day with the increased food ingestion.

The patient was admitted for further study. His PS was 2 and it had deteriorated in the last few weeks. He was overweight, although there were signs of muscular atrophy. There were signs of peripheral oedema and ascitis and a

palpable hepatomegaly of 4 cm of size. A more complete laboratory study only showed mild anemia (hemoglobin of 10.7 g/dL) and hypoalbuminemia (serum albumin of 2.2 g/dL), with no ionic abnormalities. The CEA and CA 19.9 levels had risen (448.2 ng/mL and 2155.9 UI/mL, respectively). A CT scan showed hepatic and pulmonary progression of disease; there were also signs of peritoneal carcinomatosis and ascitis and a sigmoid mass.

During his admission, the patient suffered recurrent bouts of hypoglycemia in the early morning hours which needed the use of nocturnal intravenous 10% glucose hypertonic fluid for adequate control. No thyroid or adrenal axis abnormalities were found.

An overnight fasting test was performed under close medical supervision. It was stopped at 6:00 am when hypoglycemic symptoms appeared. In that moment, the glucose level was 20 mg/dL, the insulin level was 15.5 μ UI/mL (not suppressed), the C-peptide was 5.61 ng/mL (elevated) and IGF-I was less than 2 ng/mL (suppressed). All these data were compatible with fasting hypoglycemia secondary to hyperinsulinism. Neither a pancreatic arterial and venous-phase CT scan or an octotide scan revealed signs of a primary pancreatic β -cell tumour.

Diazoxide was begun, which partially improved the hypoglycemia and the need for hypertonic fluids, at the cost of worsening of the peripheral oedemas. Due to the paraneoplastic nature of the hypoglycemia, 2nd line chemotherapy was begun with infusional 5-FU and irinotecan (FOLFIRI regimen) and a first infusion was given. However, the patients' general state quickly deteriorated and an intestinal occlusion secondary to the peritoneal carcinomatosis developed, which did not improve with medical treatment. A multiorgan failure appeared and the patient died 3 wk after the original admission. The patients' family refused an autopsy.

DISCUSSION

NICTH is a rare clinical entity. In almost half of cases it has been linked to large pleural or abdominal mesenchymal tumours^[1-3], retroperitoneal fibrosarcoma being the classic prototype. Other tumour types implicated have been hepatocarcinomas, adrenal carcinomas, and in a few cases gastrointestinal tumours, genitourinary tumours and lymphomas^[1,2,4,5]. In many cases instances the tumour is already known to be present, usually in an advanced stage^[1]. However, diagnosis can be difficult in those cases where it is the first clinical manifestation.

The pathogenesis of hypoglycemia in NICTH may involve a variety of mechanisms, including excessive consumption of glucose by what is typically a large tumour, inadequate production of counter regulatory hormones, such as growth hormone or cortisol, or ectopic or abnormal secretion of insulin or insulin-like growth factor-2 (IGF- II) and IGF-binding proteins. This last mechanism seems to be the most frequent and best characterized in patients with typical NICTH^[3-7]. Insulin secretion by the non- β -cell tumour, as in our case, is extremely rare and most cases published are secondary to secretion of an incompletely processed IGF- II by the tumour ("big IGF- II", which can be measured with specialized assays) which acts

as an insulin-like factor in the insulin receptors, causing hypoglycemia^[8]. The fasting suppression state can differentiate between both conditions, as in the setting of hypoglycemia, insulin levels will be non-suppressed in the former and suppressed in the latter. In both cases, however, IGF-I levels will be suppressed, which can be a useful marker in these patients^[9].

The clinical presentation is usually severe fasting hypoglycemia, which is persistent and requires intravenous glucose administration for reversal. Because the onset of fasting hypoglycemia is often gradual, autonomic signs are minimal or absent in most cases and neuroglycopenic symptoms predominate. They are most common in the early morning, after the overnight fast. The differential diagnosis includes all other conditions which can produce fasting hypoglycemia in adults and includes renal or hepatic failure, adrenal insufficiency, sepsis, β -cell pancreatic tumours, ethanol ingestion or drugs (usually insulin or sulfonylureas)^[9,10]. Most are easily ruled out and the differential usually only includes β -cell pancreatic tumours, adrenal insufficiency or factitious hypoglycemia secondary to exogenous administration of insulin or sulfonylureas^[10].

In our case, there were no adrenal axis abnormalities. The patient was in close medical supervision, with no contact with hypoglycemic drugs and so factitious hypoglycemia was ruled out. An overnight fast revealed a severe hypoglycemia, alongside a non-suppressed insulin and an elevated C-protein, which seemed to show an autonomous secretion of insulin. However, there were no radiological signs of a concomitant β -cell pancreatic tumour. The hypoglycemia also behaved like a paraneoplastic phenomena; its appearance was quite sudden and it coincided with the tumour progression, both radiologically and in the elevation of the tumour markers.

Treatment of this infrequent condition can be difficult. These patients often require continuous glucose infusions to control their symptoms. In some cases, diazoxide, a potent inhibitor of insulin secretion, has been useful^[1]. Debulking surgery may bring relief to the hypoglycemia, especially in those with slow-growing mesenchymal tumours^[1-3,11]. Specific treatment should be instituted if possible (e.g., imatinib in gastrointestinal stromal tumours)^[12]. In most cases, however, the outcome is often poor due to the size and advanced stage of the tumour.

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Extraction and clipping repair of a chicken bone penetrating the gastric wall

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Abstract

We report a case of gastric penetration caused by accidental ingestion of a chicken bone in a 42-year old woman with a partially wearing denture. Three days ago, she accidentally swallowed several lumps of poorly-chewed chicken. Physical examination disclosed mild tenderness in the periumbilical area. Abdominal Computed tomography (CT) showed a suspicious penetration or perforation of the stomach wall measuring about 3 cm, by a linear radiopaque material at the lesser curvature of the antrum. The end of a chicken bone was very close to but did not penetrate the liver. Endoscopic examination revealed a chicken bone that penetrated into the prepyloric antrum. The penetrating chicken bone was removed with grasping forceps. Five endoscopic clips were applied immediately at the removal site and the periumbilical pain resolved promptly. After removal of the chicken bone, the patient was treated with conservative care for three days, after which she was completely asymptomatic and discharged without complication. To treat gastric penetration by a foreign body, endocliping can be a useful method in patients with no signs or symptoms of peritoneal irritation.

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Key words: Gastric penetration; Chicken bone; Hemoclip

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INTRODUCTION

Ingestion of a foreign body is a frequent cause of injury associated with a significant morbidity and mortality. Most ingested foreign bodies pass spontaneously through the gastrointestinal tract, but some patients need endoscopic or surgical management^[1,2]. Gastric penetration by a chicken bone has been reported rarely in cases of foreign body ingestion^[3,4].

Gastrointestinal penetration by a bone fragment carries some problems in diagnosis because simple radiography is not a reliable method despite the bony calcification. Both surgical and endoscopic management are available treatments^[3-6]. The endoscopic clip, which is often used as a hemostatic procedure, has been used recently to repair perforation and close an endoscopic mucosal resection site^[7-9], and some reports on the use of a hemoclip to repair foreign body perforation are also available^[10,11].

Herein, we describe a patient with gastric penetration caused by accidental ingestion of a chicken bone, which was diagnosed using CT. The patient was treated conservatively and endoscopically by removing the chicken bone and using clipping to close the penetration site.

CASE REPORT

A 70-year old woman visited the emergency room complaining of epigastric pain for three days. She accidentally swallowed several lumps of poorly chewed chicken. She had a partially wearing denture because of teeth extraction one month ago. Physical examination disclosed mild tenderness in the periumbilical area without surgical signs including rebound tenderness. Her blood pressure was 100/60 mmHg, heart rate was 80/min, and body temperature was 36.6°C. The patient had an acute ill appearance but no history of nonsteroidal anti-inflammatory drug use, peptic ulcer, or liver disease. Laboratory studies

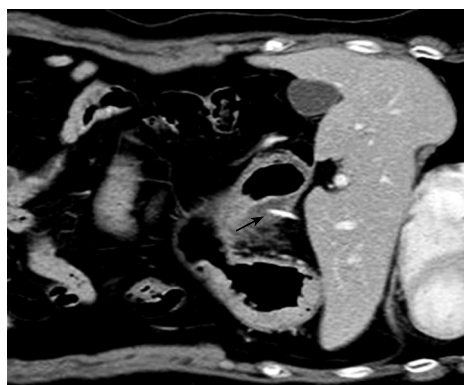


Figure 1 2.8 cm linear density (arrow) penetrating into the thickened gastric wall (CT).

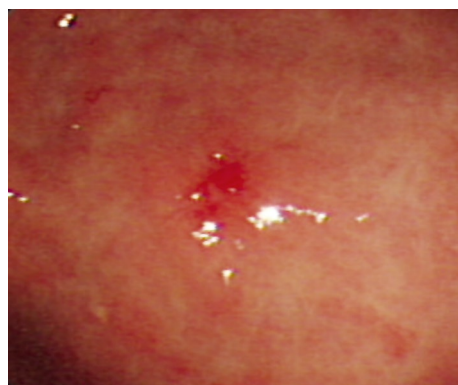


Figure 3 Follow-up endoscopy revealing near closure of the ulcer at the penetration site.

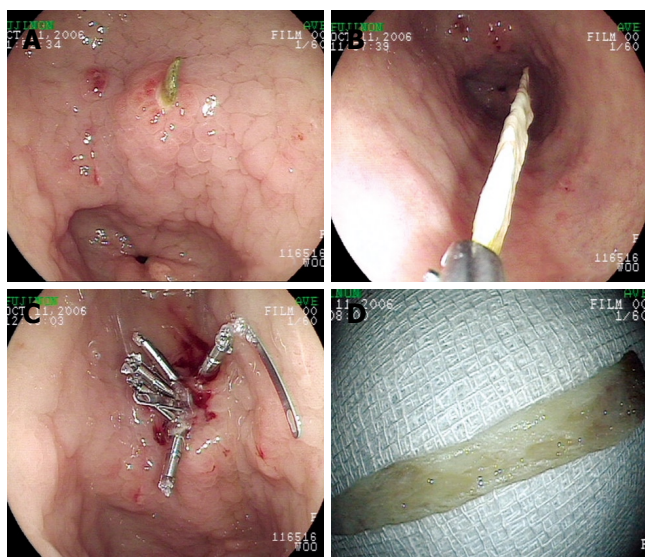


Figure 2 Endoscopic management. **A:** A chicken bone penetrating into the prepyloric antrum; **B:** The whole chicken bone was removed using a grasping forceps; **C:** Clip placement was performed at the removal site; **D:** The length of removed chicken bone was about 3.0 cm.

revealed hemoglobin concentration of 132 g/L, hematocrit 38.6%, white blood cell count of $11.7 \times 10^9/L$, and platelet count of $222 \times 10^9/L$. Her serum urea nitrogen concentration was 3.2 mmol/L, creatinine 50.4 $\mu\text{mol/L}$, AST 283 nkat/L, ALT 233 nkat/L, sodium 138 mmol/L, potassium 3.8 mmol/L, and chloride 104 mmol/L. Chest and abdomen X-ray images were negative for any abnormalities, but abdominal CT showed a suspicious penetration or perforation of the stomach wall measuring about 3 cm, by a linear radiopaque material at the lesser curvature of the antrum. The end of a chicken bone was very close to but did not penetrate the liver, and no major vessel injury was seen in the abdomen CT (Figure 1). Endoscopic examination revealed a chicken bone that penetrated into the prepyloric antrum (Figure 2A). The penetrating chicken bone was removed gently with grasping forceps (Figure 2B). No bleeding or other complications occurred after removal of the penetrating chicken bone. Five endoscopic clips were applied immediately at the removal site (Figure 2C) and the periumbilical pain resolved promptly. The removed bone fragment measured 3.0 cm in length (Figure 2D). Chest and flat abdomen X-ray imaging was performed serially after removal of the bone

and revealed no abnormalities, such as free air. After removal of the bone fragment, the patient was treated with conservative care (nothing taken by mouth, intravenous hyper-alimentation, intravenous omeprazole, and antibiotics) for 3 d, after which she was completely asymptomatic and discharged without complication. Follow-up endoscopy was performed 7 d later and showed near closure of the ulcer at the penetration site (Figure 3).

DISCUSSION

Foreign body ingestion and food bolus impaction occur commonly. Although most foreign bodies will pass out spontaneously, 10%-20% require nonoperative intervention, and 1% or fewer require surgical procedure^[1,2]. The incidence of accidental chicken bone ingestion is 6%-6.4% in Asian countries^[12,13]. There are several reports on colon wall perforation by a chicken bone treated endoscopically. Tarnasky *et al*^[14] have reported colonoscopic removal of a chicken bone impacted in the sigmoid colon without complication. Rex *et al*^[5] have reported that two patients had their chicken bones impacted in the sigmoid removed successfully by colonoscopy. However, to our knowledge, no reports are available on patients with chicken bone penetration of the gastric wall treated endoscopically with clipping and conservative care.

In adults, foreign body ingestion occurs commonly among those with prison inmates, psychiatric patients, alcoholics, children, selected professions (carpenters and dressmakers), and people wearing dentures^[15]. In our patient, the foreign body ingestion seemed to have resulted from poor dentition and an artificial denture.

Foreign bodies induce various clinical manifestations, such as perforation, bleeding, bowel obstruction, and even ureteral colic^[16,17]. A foreign body that perforates the bowel wall may take several possible courses, including lying in the bowel lumen at the site of perforation, like this patient, or passing through the gastrointestinal wall to migrate to a distal organ^[18].

In the diagnosis of nonmetallic foreign bodies (especially fish or chicken bones), CT scanning is superior to plain X-ray radiography. Plain radiography is unreliable, even with bony radiopacity, because of the masking effect of the soft tissue mass, fluid collection around the penetrated bone, and the absence of free gas in the abdomen^[6,19]. Also In this patient, CT scanning was a more reliable diagnostic method than X-ray. CT scanning showing the

calcified foreign body with a thickened intestinal segment, localized pneumoperitoneum, regional fatty infiltration, and associated intestinal obstruction is definite for diagnosis of nonmetallic foreign body perforation^[19]. However, the accuracy of CT is limited by the lack of observer awareness, and a high index of suspicion must be maintained for the correct diagnosis^[20]. Immediate surgical intervention is a traditional treatment of choice for frank gastrointestinal perforation. However, it was reported in recent years, that endoscopic clip placement, as used in a hemostatic procedure, can be used in treatment of anastomotic leaks after esophagogastric surgery and gastrointestinal perforation^[7-9]. Clipping has been used to treat foreign body removal (e.g., gastric toothpick penetration)^[11]. We decided to perform endoscopic removal of the chicken bone penetrating into the gastric wall and to use the clip to close the penetration site because CT scanning showed no peritoneal irritation and no complication associated with the bone penetration. The pain and symptoms relating to the foreign body disappeared immediately after removal of the penetrating bone fragment. However, serious complications could have occurred if the penetrating bony fragment stuck to a vessel, or if a peritoneal abscess pocket resulted from the bone penetration. Thus, it is necessary to closely observe the patient's status before and after such a procedure.

In conclusion, endoclippping can be a useful method to treat gastric penetration by foreign bodies, such as a chicken bone, in patients with no signs or symptoms of peritoneal irritation.

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CASE REPORT

Growth process of small pancreatic carcinoma: A case report with imaging observation for 22 months

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INTRODUCTION

Invasive ductal carcinoma (IDC) of the pancreas has the worst prognosis of all digestive carcinomas. Its histogenesis and natural progression are unknown, and small IDCs are still difficult to detect. This is an extremely rare case of a small IDC in which the growth process was observed on imaging studies for 22 mo.

Abstract

This report describes serial observations of the growth process of a small invasive ductal carcinoma (IDC) of the pancreas from imaging studies. Histopathological studies showed IDC with macroscopic retention cysts proximal to an intraductal papillary-mucinous adenoma with mild atypia of the branch duct type in the pancreatic body, with no relation between the two lesions. IDC was demonstrated as an extremely low-echoic mass resembling a cyst with an unclear margin on the initial endoscopic ultrasonography. We misinterpreted the low-echoic mass as a benign intraductal mucinous-papillary neoplasm (IPMN) based on findings of other imaging studies, and the patient was followed-up. The mass increased from 7 mm to 13 mm in diameter over 22 mo, and remained smaller than 10 mm in diameter for about 420 d. The tumor volume doubling time was 252 d. The Ki67 labeling index was 15.9%, similar to that described in previous reports. Hence, IDC may grow slowly while remaining small.

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Key words: Invasive ductal carcinoma; Pancreas; Intraductal papillary-mucinous neoplasm; Endoscopic ultrasonography; Tumor volume doubling time

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CASE REPORT

A 77-year-old man, who was being followed-up for chronic Hepatitis C infection, was referred to our department for evaluation of a pancreatic mass on screening transabdominal ultrasonography (US). US showed a low-echoic mass, 7 mm in diameter, in the pancreatic body, without distal dilatation of the main pancreatic duct (MPD) (Figure 1A). Endoscopic ultrasonography (EUS) demonstrated an extremely low-echoic mass with posterior echo enhancement, which appeared to be a cyst (Figure 2A). Contrast-enhanced computed tomography (CT) scan and magnetic resonance cholangiopancreatography (MRCP) revealed a grape-like cyst in the pancreatic body. Endoscopic retrograde pancreatography (ERP) indicated mucus in the MPD and a dilated branch pancreatic duct in the pancreatic body without mural nodules (Figure 3A). We misinterpreted the low-echoic mass in US/EUS images as a benign intraductal mucinous-papillary neoplasm (IPMN) of the branch duct type, and observed the lesion by US/EUS every 6 mo (Figures 1B and 2B).

Twenty-two months after the initial diagnosis, US/EUS showed the low-echoic mass had increased in diameter to 13 mm (Figures 1C and 2C). Then, for the first time, we detected a grape-like cyst distal to the lesion on US/EUS (Figure 1D), and recognized the low-echoic mass being followed was not identical to the initially diagnosed IPMN. A contrast-enhanced CT scan revealed a hypovascular area proximal to the grape-like cyst. On MRCP, the cyst did not show any change, but MPD in the pancreatic body became unclear. ERP demonstrated slight compression of the MPD proximal to a dilated branch duct (Figure 3B), and

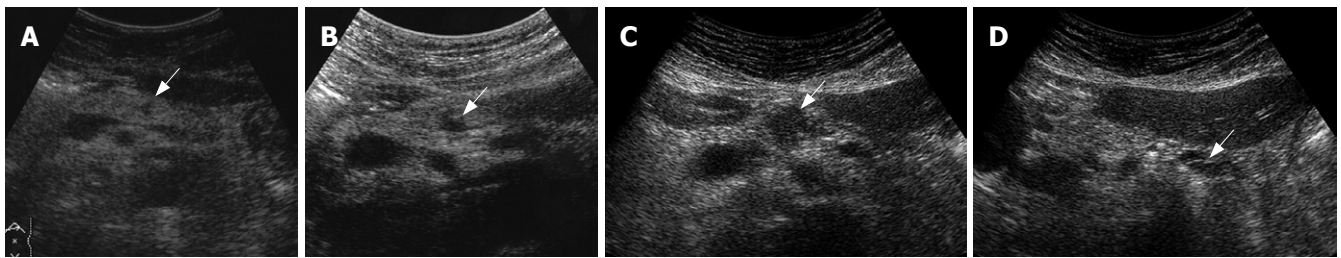


Figure 1 Chronological changes in US findings. **A:** Initial US showed a low-echoic mass, 7 mm in diameter, in the pancreatic body (arrow); **B:** After 10 mo, the diameter of the low-echoic mass had increased to 9 mm (arrow); **C:** After 22 mo, the diameter of the low-echoic mass had increased to 13 mm (arrow); **D:** After 22 mo, a grape-like cyst distal to the low-echoic mass was detected for the first time (arrow).

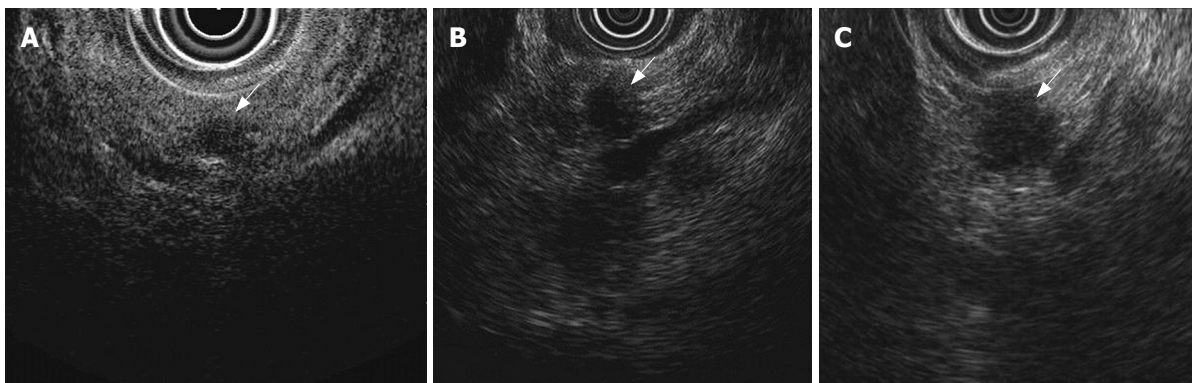


Figure 2 Chronological changes in EUS findings. **A:** Initial EUS demonstrated an extremely low-echoic mass with partial posterior echo enhancement, 7 mm in diameter, in the pancreatic body (arrow); **B:** After 14 mo, the diameter of the low-echoic mass had increased to 9 mm (arrow); **C:** After 22 mo, the diameter of the low-echoic mass had increased to 13 mm (arrow).

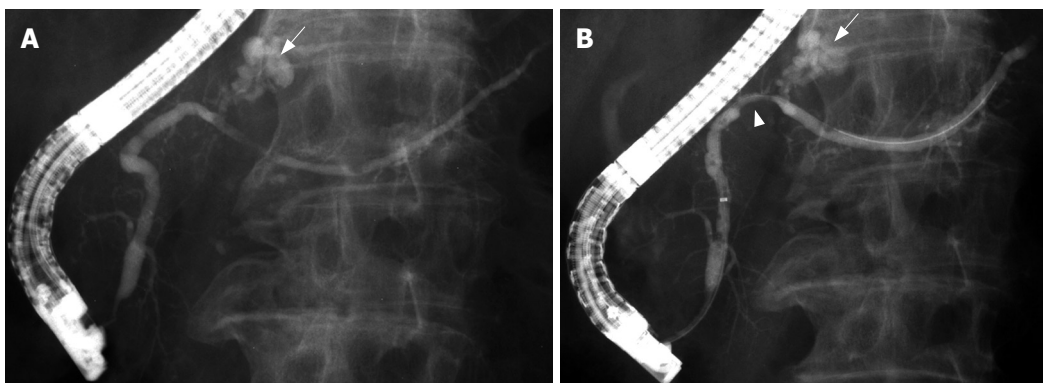


Figure 3 Chronological changes in ERP findings. **A:** Initial ERP revealed a dilated branch duct in the pancreatic body (arrow), and the MPD was mildly dilated by mucus; **B:** After 22 mo, the dilated branch duct did not show any marked change (arrow). Proximal to it, the MPD was slightly compressed (arrow head).

brush cytology did not detect any malignant cells.

Distal pancreatectomy was performed under a diagnosis of IDC concomitant with IPMN. The cut surface of the resected specimen showed a white, irregular-shaped mass with a clear margin in the pancreatic body, and a dilated branch pancreatic duct distal to the mass. Microscopic examination showed that the 13 mm × 12 mm mass was composed of moderately differentiated tubular adenocarcinoma with desmoplastic fibrosis limited to the pancreas, and included macroscopic retention cysts (Figures 4A and B). This mass was diagnosed as an ordinary IDC, not derived from IPMN, and minimal intraductal extension of IDC was seen in the MPD compressed by the mass.

A dilated branch pancreatic duct distal to the IDC was lined with low-papillary columnar cells with intracellular mucus; this was diagnosed as intraductal papillary-mucinous adenoma with mild atypia (Figure 4C). The IDC and IPMN were unrelated and were separated by normal epithelium in the MPD. After follow-up for 32 mo, our patient has shown no evidence of recurrence.

DISCUSSION

This patient demonstrated small IDC concomitant with synchronous IPMN. A branch duct type IPMN without mural nodules is a candidate for regular follow-

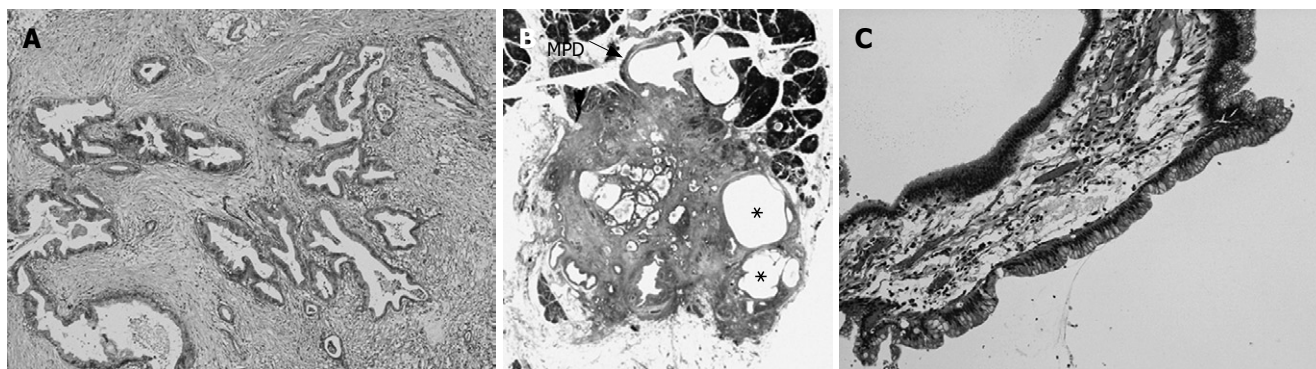


Figure 4 Histopathological findings. **A:** The mass is composed of moderately differentiated tubular adenocarcinoma with desmoplastic fibrosis, resulting in a diagnosis of IDC (HE, original magnification $\times 20$); **B:** The tumor mass includes macroscopic cystic components (*) lined by normal ductal epithelium, suggestive of retention cysts in carcinoma (HE, original magnification $\times 1$); **C:** The lining of the dilated branch duct is composed of low-papillary columnar cells with copious intracellular mucin, resulting in a diagnosis of branch duct type intraductal papillary-mucinous adenoma with mild atypia (HE, $\times 40$).

up^[1-3]. Because IPMN is sometimes superimposed on synchronous/metachronous IDC, the entire pancreas should be included in follow-up examinations for IPMN^[2,4]. In our case, a low-echoic mass seen at the initial US/EUS was misinterpreted as being identical to a cystic dilated branch pancreatic duct seen in other imaging studies, and was clinically diagnosed as a benign IPMN. Therefore, short-term observation and repeated examination were selected.

Although a small IDC is usually depicted as a solid, low-echoic mass on EUS^[5], the initial EUS in this case showed an extremely low-echoic mass resembling a cyst with an unclear margin. Histopathologically, the IDC included macroscopic retention cysts. Therefore, we considered the cyst-like mass seen at the initial EUS reflected IDC with retention cysts. It is difficult to diagnose IDC on initial imaging examination, although it is unknown whether transpapillary cytology would indicate malignant cells. However, EUS-guided fine-needle aspiration biopsy (EUS-FNAB) should be performed with caution, because cases of seeding after EUS-FNAB have been reported^[6,7]. Retrospectively, we ought to have noticed the low-echoic mass that we were following was slowly increasing in diameter, and should have selected surgical resection sooner.

In our case, the tumor volume doubling time of the IDC on US/EUS was 252 d. Furukawa *et al*^[8] have reported the tumor volume doubling time of IDC on CT scan was 159 ± 67 (median, 144) d, shorter than that in our patient. The reason for this difference may be that the initial diameter of IDCs in their study ranged from 13 to 47 mm, with a mean of 19 mm, and because the final diameter ranged from 15 to 47 mm with a mean of 30 mm, larger than that in our patient. More interesting is the fact the tumor remained smaller than 10 mm in diameter for about 420 d. The Ki67 labeling index in the present case was 15.9%, while that of the previous reports ranged from 14.5% to 29.3%^[9,10]. Hence, an IDC may grow slowly

while remaining small, although the accumulation of more cases is necessary.

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Peliosis and gummatous syphilis of the liver: A case report

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Abstract

Peliosis hepatis is a rare benign vascular disorder of the liver that may be associated with malignancy, infection and drugs. The imaging manifestation of this disorder is often variable and nonspecific making its diagnosis difficult. We describe a rare case of peliosis hepatis and gummatous syphilis of the liver with emphasis on CT findings. Image characteristics of our patient included pseudotumoral appearance of peliosis hepatis, isodensity to the adjacent liver parenchyma on unenhanced and dual-phase scanning. To our knowledge, peliosis hepatis associated with syphilis and unique enhancement pattern has not been reported. Considering the imaging features of peliosis hepatis, it should be considered in the differential diagnosis of atypical focal hepatic lesion.

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Key words: Focal liver lesion; Peliosis hepatis; Syphilis; Computed tomography

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INTRODUCTION

Peliosis hepatis is a rare kind of benign vascular disorder characterized by widespread blood-filled cystic cavities in the liver^[1]. Occasionally, the lesion mimics tumor. The imaging manifest of this disorder is variable and

nonspecific, making its diagnosis and differential diagnosis difficult. To our knowledge, no study is yet available on peliosis hepatis associated with syphilis. We report a rare case of focal peliosis hepatis and gummatous syphilis, their computed tomography (CT) and ultrasonography (US) findings along with the features of histopathology.

CASE REPORT

A 44-year-old woman had upper right abdominal pain for 4 months with no hemorrhagic tendency. Physical examination revealed no icteric skin and sclera, no abdominal mass, hepatosplenomegaly and superficial lymph nodes. On admission, treponema pallidum antibody test was positive, α -fetoprotein (AFP) was normal (1.74 ng/mL, normal range 0-8), routine blood chemistry and serum transaminase values were within the normal limits.

Abdominal ultrasonography showed a heterogeneous hypoechoic mass in the right lobe of the liver and the right hepatic vein was observed crossing the lesion with a normal shape (Figure 1). The hepatic veins, portal veins and other abdominal organs did not show any abnormalities. Unenhanced and contrast-enhanced dual-phase CT examination of the upper abdomen was performed with a 16 channel spiral CT scanner. There was an ill-defined isodensity area measuring 70 mm × 65 mm with punctate calcification in segments V and VI (Figure 2A). The attenuation of contrast in the mass was identical to that in the adjacent liver parenchyma during arterial and portal-venous phase. The center of this area was not enhanced. The right hepatic vein crossed the lesion with a normal shape (Figure 2B and C). Meanwhile, there was a hypo-attenuated lesion (10 mm in diameter) in segment VIII without enhancement at arterial and portal-venous phases (Figure 3). The spleen was normal and no lymphadenopathy was observed in the upper abdominal cavity and retroperitoneal space.

After lobectomy of the right lobe of liver was performed and the resected hepatic specimen was split, a dark violet mass was located in segments V and VI. Histopathology showed a mass with multifocal and irregular blood-filled cystic spaces and ectatic adjacent sinusoids. The histopathological diagnosis was peliosis hepatis (Figure 4), while the hypodensity lesion within segment VIII was a gumma (Figure 5).

DISCUSSION

Peliosis hepatic, first described by Wagner and named by Schoenlank, is a rare benign vascular disorder. The lesion

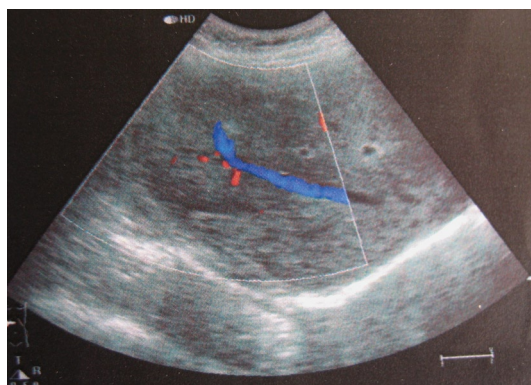


Figure 1 Doppler sonographic image showing a slightly heterogeneous hyperechoic lesion in the right lobe of liver without mass effect on the right hepatic vein.



Figure 3 Portal phase image showing a hypo-attenuated lesion (10 mm in diameter) within segment VIII (arrow). Histopathology proved it to be a gumma.

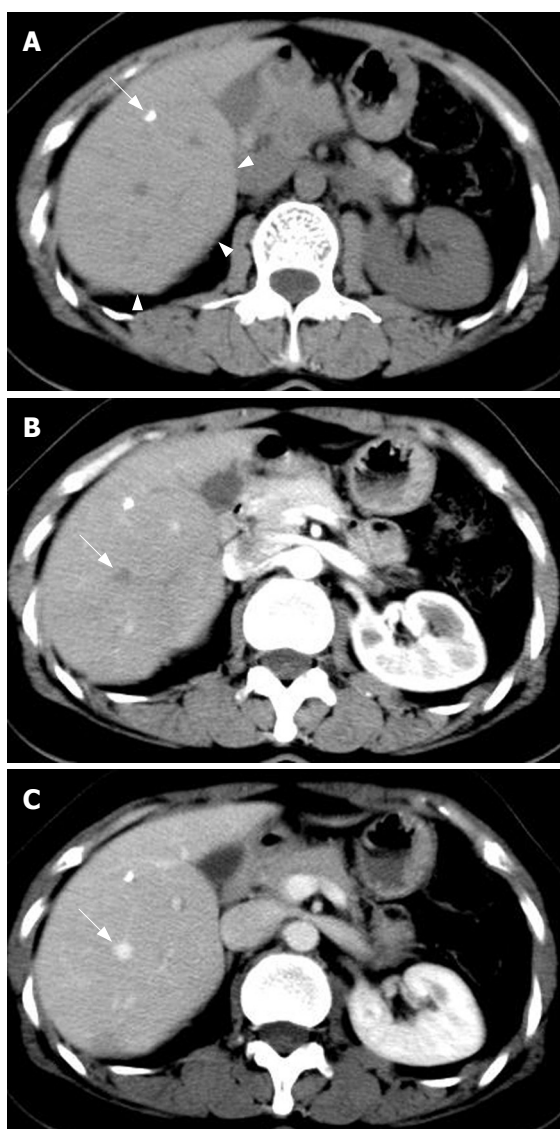


Figure 2 Transverse unenhanced CT image showing the protrudent visceral surface which hints a local iso-attenuated lesion (arrowheads) with punctate calcification (arrow) (A), identical density of the lesion to the adjacent liver parenchyma at arterial phase (B) and portal phase (C). The right hepatic vein with a normal shape and location crosses the lesion (arrow).

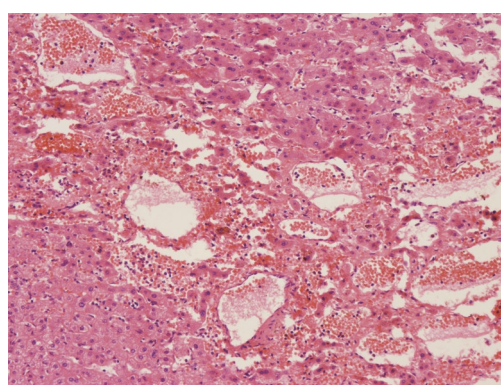


Figure 4 Histology of specimens (HE, $\times 50$). Microscopy reveals multiple blood-filled cystic spaces and ectatic sinusoids.

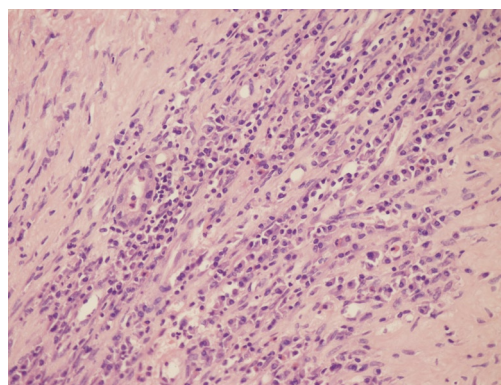


Figure 5 Gumma on segment VIII characterized by multifocal coagulation necrosis and significant infiltration of plasma cells mixed with lymph cells (HE, $\times 100$).

varies from 1 mm to several centimeters with multiple blood-filled cavities^[2,3]. The lesions typically involve the

whole liver, but local peliosis hepatis (also called peliosis hepatis pseudotumor) has also been reported^[4], like our case. Yanoff and Rawson^[5] have reported two types of the disease: parenchymal and phlebectatic. The irregularly-shaped blood spaces of the parenchymal type, usually associated with hemorrhagic parenchymal necrosis, are not lined with endothelium. The blood spaces of the phlebectatic type, based on aneurismal dilatation of the central vein, are covered with endothelium and have no hemorrhagic parenchymal necrosis.

The causes for peliosis hepatis are unknown. However,

peliosis hepatis is associated with drugs (anabolic steroids, oral contraceptives, *etc*), malignant tumor (particularly hepatocellular carcinoma), and chronic infections (pulmonary tuberculosis, leprosy and HIV infections)^[2-4]. Peliosis hepatis accompanying syphilis infection has not, to our knowledge, previously been reported. Syphilis infection may be one of the causes for peliosis hepatis, but their relationship needs to be further investigated.

The clinical manifestations and laboratory examinations of peliosis hepatis are not specific and the imaging features of US, CT and MRI may be helpful for its diagnosis. The imaging findings of peliosis hepatis are variable depending on the pathologic patterns, lesion size, extent of communication with sinusoids, and complications such as thrombosis or haemorrhage within the lesion and concomitant hepatic steatosis^[2-4]. Conventional gray-scale sonography shows hyperechoic lesions in patients with a healthy liver, homogeneous hypoechoic lesions in patients with hepatic steatosis and heterogeneous hypoechoic lesions if complicated by hemorrhage. Absence of a mass effect on peripheral blood vessels is considered characteristic of a peliosis hepatis pseudotumor^[3,4]. On unenhanced CT, peliotic lesions usually have multiple areas of low attenuation, calcifications and hemorrhage within the lesions have also been described^[2]. On MR imaging, the signal intensities of the lesions largely depend on the stage and status of the blood component. On T1-weighted sequence, the lesions are hypo-intense or heterogeneous hypo-intense if complicated by hemorrhage. On T2-weighted sequence, peliotic lesions are usually hyper-intense compared to liver parenchyma. On contrast-enhanced imaging, the lesions show a predominantly central enhancement at the arterial phase and slow centrifugal progression at the portal-venous and delayed phases (the so-called target sign) or an unusual centripetal enhancement pattern similar to hemangioma, from the periphery to the centre. Hemorrhagic parenchymal necrosis and thrombosed cavities manifest as a non-enhancing area. In some instances, small (< 2 cm) peliotic lesions also show hyper-attenuation on both arterial and

portal venous phase images^[2]. As imaging appearances and laboratory examinations are not specific, biopsy is the only way to make its diagnosis. The lesion of our case showed isodensity and was ill-defined on unenhanced scanning and synchronous enhancement with the liver parenchyma on enhanced CT. The enhancement pattern was different from previous reports. It may be due to the difference in the severity of sinusoids dilatation.

In conclusion, our case is unusual in several respects: (1) peliosis hepatis with syphilitic gumma of the liver, (2) isodensity to the adjacent liver parenchyma on unenhanced and dual-phase scanning, (3) pseudotumoral appearance. Awareness of the imaging features of peliosis hepatis is important to make its diagnosis. Peliosis hepatis should be considered in the differential diagnosis of atypical local hepatic lesion.

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www.easl.ch/hepatitis-conference

February 14-17, Berlin, Germany
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www.kenes.com/immuno

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

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 British Society of Gastroenterology Annual Meeting
 E-mail: BSG@mailbox.ulcc.ac.uk

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

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

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

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

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

April 23-27, Milan, Italy
 43rd Annual Meeting of the European

Association for the Study of the Liver
www.easl.ch

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

May 18-21, San Diego, California, USA
 Digestive Disease Week 2008
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www.congrex.com/ngc2008
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 Email: meetings@imedex.com

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^[1]Passed away on October 20, 2007

^[2]Passed away on June 11, 2007

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Wireless capsule endoscopy

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Abstract

Wireless capsule endoscopy is a new technique that allows complete exploration of the small bowel without external wires. Its role has been analyzed in many small bowel diseases such as obscure gastrointestinal bleeding, Crohn's disease and gastrointestinal polyposis syndromes with promising results. Studies on other pathologies (i.e. small bowel tumour, celiac disease) are under evaluation to define the role of this technique.

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Key words: Wireless capsule endoscopy; Small bowel; Obscure gastrointestinal bleeding; Crohn's disease; Gastrointestinal polyposis syndrome

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The need and wish to perform endoscopic examination of the small bowel have led to the development of an ingestible miniature camera device capable of obtaining images of the whole small intestine.

Wireless capsule endoscopy is a new type of radiotelemetry video system which is small enough to be swallowed and has no external wires, fiberoptic bundles or cables. It measures 11 mm × 26 mm and weighs 3.7 g. By using a lens of a short focal length, images are obtained as the optical window of the capsule sweeps past the gut wall, without requiring air insufflation of the gut lumen. The capsule is propelled by peristalsis through the gastrointestinal tract and does not require a pushing force to propel it

through the bowel. Up to 2002, more than 250 000 capsule explorations had been performed^[1], and nowadays this number has increased significantly.

The M2A capsule [Mouth to (2) Anus] initially, and Pillcam SB (Small Bowel) later, from GIVEN (GastroIntestinal Video Endoscopy, Given Imaging Limited, Yoqneam, Israel), and endocapsule from Olympus are the capsules that have been approved for use in the clinical setting. Each capsule contains a lens, light emitting diodes (LEDs), a color camera, 2 batteries, a radio frequency transmitter and an antenna. The camera takes 2 images per second and transmits these by means of radio frequency to a sensor array in a belt placed around the patient's abdomen and from there to a recording device in the belt. Once the study is completed (between 6 and 8 h), the recording device is removed and the images are downloaded to a computer workstation with software that displays the video images on a computer monitor.

Capsule endoscopy can be performed as an outpatient procedure. Small bowel preparation is still a controversial issue. Some groups used fasting or clear liquids for 10 to 12 h (or even for 24) before the study, although some studies suggest that bowel preparation (with 2 or 4 litres of polyethylene glycol based electrolyte solution or oral sodium phosphate preparation) improves the visualization of the small intestine^[2,3]. A recent Spanish prospective multicenter trial published in abstract form, has shown that all three strategies have similar results^[4].

The role of wireless capsule endoscopy has been analyzed in patients with obscure gastrointestinal bleeding and in comparative studies with endoscopic^[5] or radiographic methods^[6]. Capsule endoscopy has shown a diagnostic yield of 71% compared to 29% of push enteroscopy, in a recent analysis of 7 prospective studies^[7]. Another study has shown that the detection rate of capsule endoscopy is higher in patients with ongoing overt bleeding than in those with anemia or prior overt bleeding^[8]. In a comparative study with intraoperative enteroscopy, the sensitivity, specificity, positive and negative predictive value of capsule endoscopy were 95%, 75%, 95% and 86%, respectively^[9]. For obscure gastrointestinal bleeding, capsule endoscopy has shown better results than radiographic studies, which have a low diagnostic yield in detecting small bowel lesions^[6,10].

Capsule endoscopy has also shown its usefulness in the evaluation of the small intestine in patients with suspected or known Crohn's disease^[11], and is superior to small bowel follow-through^[12-14], enteroclysis^[15,16], push enteroscopy^[16] and CT enteroclysis^[17] for identifying small intestinal dis-

ease. The sensitivity and specificity of capsule endoscopy have recently been estimated to be 89.6% and 100%, respectively^[18].

However, the diagnostic criteria of capsule endoscopy for Crohn's disease have not yet been defined. Mucosal breaks and aphthous ulcers or erosions are also seen in asymptomatic healthy volunteers, and small bowel ulcers and strictures have been associated with the use of nonsteroidal anti-inflammatory agents, making it, at times, difficult to differentiate these findings with the presence of a Crohn's disease^[9].

Capsule endoscopy has been performed in patients with gastrointestinal polyposis syndrome, and several studies have suggested that it may be useful in the detection of small bowel polyps^[19,20]. A comparative prospective study showed that capsule endoscopy can detect more polypoid lesions than small-bowel follow through in these patients^[21]. Nevertheless, more prospective studies with longer follow-up are required, to define the role of capsule endoscopy findings in the outcome of patients with gastrointestinal polyposis syndrome.

Capsule endoscopy in the pediatric population and esophageal capsule endoscopy (Pillcam ESO) have shown promising results but larger prospective trials are needed to define their role in these patients.

Other possible indications for capsule endoscopy, such as celiac disease, HIV positive patients with gastrointestinal symptoms, mal-absorption or small bowel transplantation, have not been defined so far, and more prospective trials assessing the use of capsule endoscopy in these groups of patients are still needed.

The main contraindication of performing the capsule endoscopy procedure is the suspicion or knowledge of a gastrointestinal obstruction, stricture or fistula. Other former contraindications such as implanted cardiac pacemakers or other electro-medical devices and patients with swallowing disorders have been excluded since some studies showed no interference between capsule endoscopy and pacemaker or implantable defibrillators functioning^[22,23] and endoscopic placement of the capsule into the gut^[24].

The capsule retention rate varies with the indication of the examination, being reported of 1.5% in patients with obscure gastrointestinal bleeding^[25] and 5% in patients with suspected Crohn's disease^[26], who are usually asymptomatic^[10,25] and may require endoscopic removal or surgery. How to prevent capsule retention has yet to be defined since neither radiologic studies nor the "patency capsule" has shown conclusive results so far. The clinical setting of each patient, as well as some features related to intestinal strictures (previous small bowel surgery, NSAIDs, suspected small bowel Crohn's disease), have to be analyzed prior to the study. Patients should be informed about the possibility of capsule retention and further treatment.

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EDITORIAL

Current view of the immunopathogenesis in inflammatory bowel disease and its implications for therapy

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Abstract

Although the aetiology of inflammatory bowel disease (IBD) remains unknown, the pathogenesis is gradually being unravelled, seeming to be the result of a combination of environmental, genetic, and immunological factors in which an uncontrolled immune response within the intestinal lumen leads to inflammation in genetically predisposed individuals. Multifactorial evidence suggests that a defect of innate immune response to microbial agents is involved in IBD. This editorial outlines the immunopathogenesis of IBD and their current and future therapy. We present IBD as a result of dysregulated mucosal response in the intestinal wall facilitated by defects in epithelial barrier function and the mucosal immune system with excessive production of cytokines growth factors, adhesion molecules, and reactive oxygen metabolites, resulting in tissue injury. Established and evolving therapies are discussed in the second part of this editorial and at the end of this section we review new therapies to modulate the immune system in patients with IBD.

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Key words: Inflammatory bowel disease; Ulcerative colitis; Crohn's disease; Tolerance; Cytokines; Mucosal inflammation

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Torres MI, Ríos A. Current view of the immunopathogenesis in inflammatory bowel disease and its implications for therapy. *World J Gastroenterol* 2008; 14(13): 1972-1980 Available from: URL: <http://www.wjgnet.com/1007-9327/14/1972.asp> DOI: <http://dx.doi.org/10.3748/wjg.14.1972>

INTRODUCTION

The intestinal mucosa constitutes an immunologic system in which oral tolerance and defence against harmful organisms develop^[1]. The mucosal immune system, which plays a pivotal role in host defence, is continuously exposed to large amounts of exogenous (i.e., dietary) and endogenous (e.g., bacterial) antigens^[2]. Activation of lamina propria (LP) T cells by lumen antigens may lead to the production of inflammatory cytokines and subsequent mucosal inflammation and tissue damage^[3,4]. Dysfunctions of the intestinal immune system and cross-reactivity against host epithelial cells have been implicated as major mechanisms by which inflammation occurs^[5]. In addition to immune factors as leukocytes, macrophages, polymorphonuclear cells, mast cells, B and T lymphocytes, other potential mediators of inflammation are involved in the disease: eicosanoids, biological amines, cytokines, nitrogen- and oxygen-reactive metabolites, platelet-activating factor have been noted to influence inflammatory processes^[6]. Among environmental factors, antigens to intestinal bacteria play an important role. The loss of tolerance to intestinal bacterial flora is manifested as an abnormal immune response^[7]. Accordingly, inflammatory bowel disease (IBD) could be considered to be an imbalance between pro-inflammatory and anti-inflammatory mediators^[3]. The following editorial summarizes the current view of the immunopathogenesis basis of IBD in relation to barrier disruption, immune deregulation genetic epidemiology and susceptibility to environmental dysregulation, triggers and its implications for therapies.

EPIDEMIOLOGY AND CLINICAL FEATURES

IBD like ulcerative colitis (UC) and Crohn's disease (CD), comprises a group of multifactorial intestinal disorders with high incidence throughout the human population^[8]. The highest incidence rates and prevalence have been reported in northern Europe and North America. The disease is characterized by cycles of clinical exacerbation and remission, with periods of improvement followed by relapse, and appears to be immunologically mediated^[12]. UC and CD show some overlapping clinical features, and in 10%-15% of cases it is not possible to differentiate between the two diseases. But also, differences exist in the nature and location of the lesion damage between UC and CD. While UC is restricted to the large intestine and is associated with continuous mucosal inflammation, including crypt abscesses as well as ulcers, which typically

Table 1 IBD immunopathogenesis

Immune dysregulation
Cytokines
Epithelial barrier function
Oral tolerance
Reactive oxygen species (ROS)
Antioxidant defence system

spread from the most caudal part of the rectum, CD can affect any part of the gastrointestinal tract and is characterized by segmental and transmural inflammation, fistulas, oedema and granulomas in whole intestinal wall^[13].

At the histological level, various abnormalities have been observed in patients with IBD and in experimental models of intestinal inflammation including the presence of a significant number of neutrophils within the lamina propria and the crypts, where micro-abscesses can form, the depletion of goblet cell mucin, induced by disturbed transformation of undifferentiated cells in an environment exposed to inflammatory cytokines, damage to the nervous system^[13,14] and hypertrophy of smooth muscle cells^[15]. Intestinal inflammation alters the contractile activity of intestinal smooth muscle, and the motility disorders induce abnormal growth of the intestinal flora, resulting in disturbance of the intestinal flora that can aggravate the pathogenesis of mucosal inflammation^[16].

GENETIC FACTORS

The discovery of susceptibility genes to IBD have shown the importance of (1) dysregulation of the innate immune response to enteric microflora or pathogens; (2) increased permeability across the epithelial barrier, and (3) defective regulation of the adaptive immune system of epithelial barrier function in disease pathogenesis. IBD is considered a complex polygenic disease and its susceptibility genes could interfere with the disease and with the response to different therapies.

Strong evidence for genetic factors has been reported contributing to IBD susceptibility^[17]. IBD is thought to be caused by the mutual reactions among host susceptibility genes (CARD15/NOD2, CARD4/NOD1, HLA, TLR4, DLG5, NF- κ B1), environmental factors including enteric flora and food antigens, and abnormal immune balance^[9-11]. The genetic contribution may be more important in CD than UC with multiple gene products contributing to risk^[18]. Identifying NOD2 as a susceptibility gene in CD and additional susceptibility loci have been implicated in IBD such as IBD5, IL23R and ATG16L1 among other loci^[19-21]. The number of potential gene continues to increase, additional novel loci map to chromosomes 16q24.1, TNFSF15, NKX2-3 and the intergenic region on chromosome 10q21.1 might contribute to the IBD^[22]. Future genetic research may include focus on phenotypes, control for environmental variables and gene-gene interactions.

PATHOGENESIS OF IBD

There is compelling evidence that dysregulation of the

mucosal immune system is a major factor contributing to the pathogenesis of IBD^[13]. The pathogenesis of these diseases is understood to represent the outcome of three essential, interactive cofactors: host susceptibility, enteric microflora and mucosal immunity^[12,13]. The basis of IBD is the presence of genetically determined alterations that result in a mucosal immune system that overreacts to normal intestinal microflora. These immune responses may be induced by defects in the epithelial barrier, and increased intestinal permeability, adherence of bacteria, and decreased expression of defensins^[12].

The mucosal immune system senses and interprets the local microenvironment, recognizing and avoiding reactions to commensal flora (tolerance), whilst retaining its capacity to respond to episodic challenge from pathogens^[1,2]. Increased synthesis and release of pro-inflammatory mediators such as cytokines, chemokines, eicosanoids, platelet activating factor, reactive oxygen and nitrogen metabolites, as well as other abnormalities have been in IBD^[6]. We described IBD as a result of dysregulated mucosal response in the intestinal wall facilitated by defects in epithelial barrier function and mucosal immune system with excessive production of cytokines growth factors, adhesion molecules, and reactive oxygen metabolites, resulting in tissue injury (Table 1).

DYSREGULATED IMMUNITY

Cytokines

Alterations in the production of many cytokines have been described in patients with active IBD^[23]. The significance of these findings in the pathogenesis of IBD remains poorly understood and controversy has continued as to whether these changes really represent a primary defect in the regulation of the immune system or are a secondary consequence of immune activation^[6].

The nature of the immune response and the cytokine profile generated are under genetic control and determine the features of the inflammatory process in IBD. In active IBD the balance of regulatory and effector cells is disturbed, predominating effector T cells (Th1, Th2) over regulatory T cells (Th3, Tr). CD has been associated with type 1 helper T-cell cytokines (Th1), such as interferon- γ , TNF- α and IL-12^[24,25]. In ulcerative colitis, the pattern of cytokine is less clear; there is a modified Th2 response associated with cytokines such as IL-15 and IL-10^[25,27]. Among the cytokines involved, Interleukin-10 is a regulatory cytokine which plays a crucial role in the balance of the mucosal immune system, promoting physiological activation and preventing the pathological inflammation that characterizes the inflammatory bowel diseases^[26]. The immuno-regulatory activity of IL-10 is based upon its ability to inhibit both cytokine synthesis and antigen presentation, with maintenance of intestinal immuno-regulation and tolerance to components of intestinal flora, controlling the inflammatory responses to intestinal antigens^[26].

This pathophysiological concept for IBD is changing as a result of recent advances with the description of another type of effector immunologic response CD4+ Th pathway, namely interleukin-23/interleukin-17 axis. IL-23

is produced by antigen-presentation cells and promotes a population of T lymphocytes that produce IL-17, IL-6 and TNF- α (Th17 cells)^[27,28]. These IL-23-driven CD4⁺ T cells are highly inflammatory and elicit IL-17-dependent autoimmunity. IL-17 is an inflammatory cytokine expressed by activated CD4⁺ T cells and triggers the NF- κ B signalling cascade and MAP kinase pathway, leading to T cells proliferation and up-regulation of inflammatory molecules^[29]. Recent publications have reported that the IL-23/IL-17 pathway may have a pivotal role in intestinal inflammation^[30,31]. These findings demonstrate that IL-23, but not IL-12, is essential for the development of intestinal inflammation in IBD. In addition, levels of expression of IL-23 and IL-17 are increased in patients with CD^[32]. Recently, the study of Duerr *et al*^[33] reported a significant association between CD and a gene on chromosome 1p31 that encodes a receptor for IL-23 (*IL-23R*) that is highly expressed by memory T cells.

Also, the interesting works of Bamias *et al*^[34] and Prehn *et al*^[35], respectively, have described a novel TNF-like cytokine co-stimulator of IFN- γ , namely TL1A that play an important role in mucosal inflammation and in experimental ileitis. TL1A is expressed in dendritic cells and acts on CD4⁺ cells and provides co-stimulation for proliferation of IFN- γ and is up-regulated in patients with active IBD. A single nucleotide polymorphism in the gene encoding TL1A (*TNFSF15*) confers susceptibility to CD^[36].

Mucosal epithelial barrier function

The polarized epithelial cells provide a crucial barrier function, with high concentrations of dietary and bacterial antigens at the apical surfaces in the mucosa and with high concentrations of lymphoid cells at the basolateral surface^[37]. The intestinal epithelium is considered a constitutive component of the mucosal immune system. Intestinal epithelial cells (IECs) are connected by tight junctions, which are dynamically regulated in response to cytokines and are down-regulated by the junctional complexes in human IBD (E-cadherin and β -catenin)^[38]. The epithelium is in constant communication with luminal flora and the underlying network of innate and adaptive immune cells, and IECs constitutively express or can be induced to express costimulatory molecules^[39] and components of the human major histocompatibility complex (MHC)^[40], toll-like receptors (TLRs)^[41], NOD proteins^[42], inflammatory cytokines^[43], as well as antimicrobial peptides^[44].

IECs contribute to the initiation and regulation of innate and adaptive defence mechanisms by interacting with lamina propria dendritic cells (DC), lamina propria lymphocytes (LPL), intraepithelial lymphocytes (IEL) and mediators of the immune and the enteric nerve system^[45]. IECs such as non-professional antigen-presenting cells might interact with naïve T cells (Th0) through MHC II receptors, and produce co-stimulatory signals suppressing or inducing anergy in mucosal T cells^[46]. The epithelial barrier is leaky in IBD and has a lower epithelial resistance and increased permeability that enables the proliferation of non-pathologic organisms (normal microflora) in close juxtaposition to elements of the mucosal immune system.

Also, IBD have disturbed innate immune mechanisms of the epithelial layer and mucosal epithelial cells have a different pattern of TLR expression^[47]. Also, an up-regulation of NOD2 in IECs and disturb in antigen recognition and processing by antigen-presenting cells are present in patients with IBD^[48].

Paneth cells secrete antimicrobial peptides, including α -defensins that play an important role in innate intestinal defences. The Paneth cell deficiency in α -defensins increases the risk of CD and polymorphism in the defensin gene associated with CD^[49].

Goblet cells are also an important component of the epithelium and are responsible for defence and epithelial mucosal repair in colitis. Defects in mucus production have been reported in IBD^[50,51].

The adaptive immune response in IBD failure to balance, and the DCs might be responsible for a dysregulated innate immune response^[52]. DCs are the primordial cells in controlling immunity against pathogens and tolerance towards commensals and are dominant subsets of antigen-presenting cells in the intestinal lamina propria^[53]. DCs penetrate their dendrites between epithelial cells to sample luminal antigens without altering the mucosal barrier and they contain components of commensal bacteria. Depending upon the nature of the antigen and the activation state of DCs, the end result may be immune activation or tolerogenic action^[54]. DCs also have an important role in mucosal inflammation through the production of cytokines, resulting in persistent activation of effector T cells^[55].

An increase in intraepithelial lymphocytes (IEL) appeared in IBD and the activation of these IELs could be responsible for: (1) defending the mucosal barrier against intraluminal microorganisms, (2) modulation on epithelial cells of the expression of MHC antigens, or (3) non-MHC as well as MHC-restricted cytotoxicity^[56]. The increase of IELs is known to be associated with an abnormal expression of class II MHC molecules on surface and crypt colon epithelial cells. Helper T cells could trigger an MHC-restricted immune mechanism.

Oral tolerance

In an antigenic environment like intestine, the mucosal immune system must maintain tolerance to commensal bacteria, food and self antigens and must be able to initiate defence responses to pathogens. Studies have suggested that IBD is a consequence of the breakdown of mucosal tolerance and tolerance to normal flora is broken^[57]. DCs in conjunction with antigen-specific T lymphocytes trigger the maintenance of immune tolerance. Activation of intestinal NKT cells by CD1d-expressing IECs and professional antigen presenting cells (DC cells) may contribute to induction of oral tolerance^[58,59].

In IBD, atypical antigen-presenting cells become potent effector-T-cell activators, and the IECs can act as antigen-presenting cells capable of stimulating primed T cells which acquire an activated phenotype with increased histocompatibility molecule expression in the presence of inflammatory cytokines^[60]. IECs might also activate T cells *via* non-classical MHC. Our research group has previously shown that intestinal epithelial cells expressing HLA-G at

Table 2 Medical management based on pathogenesis

Dietary nutrients
Polyunsaturated fatty acids (PUFAs)
Fiber
Probiotics
Prebiotics
Flavonoids
Antagonist platelet-activating factor (PAFs)
Biological therapies
Anti-inflammatory/Immunosuppressive
Immunomodulators
Generation regulatory T cells/Activation effector T cells
Inhibition of recruitment, migration, adhesion molecules
Epithelial repair/Restitution barrier function
Induction apoptosis
Autologous hematopoietic stem cell transplant

the apical surface of the epithelial layer and at crypt level in ulcerative colitis, but not in CD^[61]. HLA-G is a non-classical major histocompatibility complex class I, which is selectively expressed at the maternal-foetal interface of cytotrophoblast cells and protects the foetus from maternal rejection and creating a general state of tolerance^[62].

Non-typical antigen-presenting cells, as epithelial cells, might acquire an activated phenotype with increased expression of HLA-G in the presence of inflammatory cytokines as IFN-gamma and TNF-alpha and might activate T cells *via* non-classical MHC molecules as HLA-G^[61]. In UC the specific expression of HLA-G, considered as a molecule implicated in tolerance immunity will be of great importance in the maintenance of the tolerance to fed antigens and of gut microflora and support the notion that it may serve as a regulator of mucosal immune responses to antigens of undefined origin.

REACTIVE OXYGEN SPECIES (ROS)

The intestinal mucosa is vulnerable to oxidative stress from constant exposure to reactive oxygen species (ROS) generated by the lumen contents such as oxidized food debris, transition metals such as iron and copper, bacterial metabolites, bile acids and salivary oxidants^[63]. A balance is maintained between oxidant and antioxidant systems under physiological conditions, but it is impaired in pathological success. Oxidant-mediated injury plays an important role in the pathophysiology of IBD^[64]. It has been suggested that intestinal damage in IBD is related to increased free radical production and to impaired antioxidant defence systems^[65]. There are an increased number of activated inflammatory cells in lamina propria of IBD releasing reactive oxygen radicals that are highly produced by neutrophils, macrophages and DCs^[63], and exceeds the limited intestinal antioxidant defence system, contributing to intestinal oxidative injury in IBD^[64].

Our research group has undertaken to determine whether the small intestine is subjected to oxidative damage during experimental IBD induced by administration of TNBS, as well as, to examine the accompanying changes in antioxidant status, in order to understand its role in the pathogenesis of disease.

TNBS may be metabolized to yield superoxide anion (O_2^-), and hydrogen peroxide (H_2O_2), suggesting that TNBS-induced intestinal inflammation may be partially mediated by cytotoxic reactive oxygen metabolites generated by the oxidative metabolism of TNBS^[66]. We evaluated the activity of the antioxidant enzymes catalase (CAT), glutathione peroxidase (GPX), glutathione reductase (GR), glutathione transferase (GT) and superoxide dismutase (SOD), as well as the levels of total glutathione (GSH). We found that GSH levels and SOD activity decreased in animals administered TNBS. These result are in agreement with studies in human IBD, that have been reported a decreased superoxide dismutase activity, as well as low total glutathione levels^[67]. Decreased GSH in gut epithelial cells may increase susceptibility to oxidative injury and exacerbate degeneration of the intestinal mucosa^[28]. Therefore, the elevated activities of glutathione peroxidase (GPX) and catalase (CAT) enzymes observed suggest that TNBS led to the formation of high levels of peroxides, including H_2O_2 , increasing tissue injury^[68].

Nutritional deficiencies have been reported in IBD, such as lower levels of vitamins A, E and C, important natural antioxidants for the organism, as well as a decrease in trace elements such as zinc and selenium, which are crucial components of several antioxidant enzymes such as SOD^[69].

MEDICAL MANAGEMENT BASED ON PATHOGENESIS

The main goal of therapy for IBD is to induce a clinical remission and then maintain it for a long period of time, in order to realize the best attainable quality of life. Choice of therapy depends on the severity and location of disease, as well as side effects and other adverse events. Although 5-aminosalicylates, corticosteroids and immunosuppressive drugs are generally used in the treatment of IBD, there are an important numbers of the patients who are not controlled by these conventional therapies^[70], indicating a considerable need for develop new and more effective therapy. Treatment with anti-diarrhoeal agents, proper nutrition, antagonistics of activation platelets factors, flavonoids, probiotics and prebiotics can be efficient, but also, a growing number of new biological agents are under investigation, as monoclonal antibodies to antisense mRNA products, peptides and vaccines among others. In Table 2 we summarised medical management based on pathogenesis.

Dietary nutrients

Dietary management of IBD may be an interesting alternative to drug therapy if it proves to be effective without side effects and can be used as a remission induction and maintenance therapy^[71]. Nutrients may be involved in the modulation of the immune response, thus, as components of cell membranes, nutrients can mediate the expression of proteins involved in the immune response, such as cytokines, adhesion molecules, *etc*^[71].

A potential relationship between components of the

diet and disease pathophysiology has been considered, and immunologic mechanisms have been postulated to link food antigens and the development of intestinal inflammation. Some reports have suggested that consumption of refined sugar might be a risk factor for CD, but not UC^[72]. Fat intake has been reported to be positively associated with UC, whereas fruit, vegetables, and fiber consumption seem to decrease the risk of IBD^[73].

Polyunsaturated fatty acids (PUFAs)

The composition of lipids in the cell membrane is modified by dietary changes and can influence cellular responses. Dietary lipids are one of the most active nutritional substrates modulating the immune response. The link between fatty acids and inflammation is that the eicosanoid family of inflammatory mediators is generated from PUFAs^[74]. Long chain n-3 PUFAs decrease the production of inflammatory eicosanoids, cytokines and adhesion molecules, inhibiting the arachidonic acid metabolism and altering the expression of inflammatory genes across effects on transcription factor activation and intraluminal bacterial content^[75].

Several studies have shown that patients with IBD had an abnormal plasma phospholipids fatty acid profile^[76,77]. The loss of the omega-3 fatty acids which increases the n-6. n-3 ratio, would lead to a predominance of proinflammatory eicosanoids. In an experimental model of IBD induced by rectal administration of TNBS when the animals received dietary n-3 PUFAs, they showed beneficial effect by competing with n-6 PUFAs for the production of lipid inflammatory mediators such as leukotrienes (LTB₄), thromboxane (TXA₂), Prostaglandins (PGE₂) and cytokines^[77]. The restoration of GSH levels after administration of the n-3 PUFAs demonstrated the decrease in oxidative stress. It was suggested that dietary PUFAs could affect mucosal adhesion sites for gastrointestinal bacteria by modifying the composition of the intestinal wall and as a result, the dietary PUFAs could modulate the probiotics action^[78].

Although LC-PUFA may be of interest in the dietary management of IBD, these fatty acids are highly susceptible to peroxidation, and indeed they may influence the antioxidant defence system. To overcome lipid peroxidation by products and cell damage from diets supplemented with PUFAs, appropriate antioxidants should be provided.

Dietary fiber

Several studies have shown that dietary fiber actively contributes to the intestinal anti-inflammatory effect, supporting its potential role in the treatment of IBD. Their therapeutic effect is associated with an increased production of short-chain fatty acids, mainly acetate, propionate and butyrate in the colonic lumen and with the promotion the use of these fatty acids, specifically butyrate by colonic epithelial cells^[79]. The result is the restoration of the metabolic function of the intestinal cells by aerobic ATP production after butyrate oxidation by epithelial cells that accelerate the intestinal repair preserving the integrity of the intestinal mucosa and downregulating the exacerbated immune response presented in IBD^[80].

The dietary fiber associated with the production of short-chain fatty acids, also can contribute to the inhibition of the production and release of proinflammatory cytokines, including IL-6, IL-8, TNF-alpha, and other mediators of inflammation as reactive oxygen and nitrogen metabolites (NO production)^[81].

Probiotics and prebiotics

IBD represent a malfunction of tolerance to the commensal microbiota, for this reason attention is focused on this relationship as a potential therapeutic action. Therapies such as prebiotic and probiotics that selectively manipulate the gastrointestinal microbiota present an attractive treatment in IBD with maintenance of remission and without major side effects. Some evidences support that the use of probiotics and prebiotics in IBD need to use standardized methodology to confirm their utilization as therapy^[82].

Probiotics

The human intestine contains different bacterial species, named intestinal microflora that plays a critical role in maintaining intestinal health. This intestinal microflora protects against pathogens and maintains the epithelial barrier integrity^[83]. The functions of these bacteria include control of proliferation and differentiation of IECs and development and balance of the immune system.

Probiotics are living microorganisms that upon ingestion in specific numbers have beneficial effect and exert their therapeutic effects to modulate the barrier function, the inhibition of pathogenic bacteria, the intestinal production of cytokines, with anti-inflammatory properties and enhancement of the digestion and absorption of food^[83,84].

Prebiotics

Prebiotics are selectively fermented short-chain carbohydrates that allow specific changes in the composition and activity of the microbiota in the gastrointestinal tract and confers health benefits, these include fructo-oligosaccharides and galacto-oligosaccharide^[85,86]. Prebiotics have been shown to enhance the immunoregulatory bacteria of lumen, to reduce the activity of pro-inflammatory transcription factors and attenuate the inflammation. In this sense prebiotics produce butyrate and acetate that inhibit, mucosal inflammation, acting on epithelial and DCs function^[87].

Flavonoids

Flavonoids are polyphenolic compounds that occur ubiquitously in foods of plant origin and exert antimicrobial, antiviral, antineoplastic, antihepatotoxic, hypolipidemic, antiallergic and anti-inflammatory features^[88]. Biochemical investigations of flavonoid mechanisms have demonstrated that these compounds inhibit a wide variety of enzymatic systems. Variations in the heterocyclic ring C give rise to flavonols, such as morin.

Morin (2',3,4',5,7-pentahydroxyflavone, flavonol) is a yellowish pigment extractable from the wood of *Chlorophora tinctoria* and acts as a broad-spectrum and non-toxic antioxidant^[89]. Our laboratory analyzed the effects of morin on experimental TNBS-induced IBD

in rats. Oral administration of morin, at doses ranging from 10 to 200 mg/kg, significantly reduced the mucosal damage by 20%-40% induced by the TNBS experimental model of IBD, although these beneficial effects were not dose-related. Morin reduced the enzymatic activity of myeloperoxidase (MPO) (marker of neutrophil infiltration) and can be interpreted as a manifestation of this anti-inflammatory property^[89]. Also, significantly inhibited LTB₄ synthesis, and this inhibition was maximal at the highest dose of morin assayed (200 mg/kg). Also, morin reduces colon oxidative stress induced in the TNBS model. This reduction may be explained on the basis of its ability both to inhibit free-radical production and to scavenge free radicals once they have been released and by inhibition of colon LTB₄ synthesis.

Antagonistic platelet-activating factor

Sulphasalazine, a diazo compound with 5-aminosalicylic acid (5-ASA) linked to sulphapyridine that acts as a carrier, has been used as therapy for IBD. This agent is useful in maintaining remission by prevention of relapses in patients with IBD^[90]. Unfortunately, long-term administration of sulphasalazine is accompanied by a considerable number of side-effects, either dose-dependent such as nausea, vomiting, headache, *etc.*, or allergic such as cutaneous rashes, exanthema, fever, *etc.*

Several mechanisms have been postulated as being involved in the intestinal anti-inflammatory effect exerted by 5-ASA derivatives, including antioxidant and/or radical scavenging properties, inhibition of leukocyte chemotaxis and inhibition of IL-1 synthesis^[91]. It has been shown that colon mucosa from patients with IBD produces high levels of platelet-activating factor (PAF) with an important role in the pathogenesis of IBD, its inhibition by specific antagonists may have a potential therapeutic benefit in the treatment and management of these inflammatory diseases^[91].

UR-12746 is a compound which combines 5-ASA and UR-12715 through an azo link, and possesses antagonist PAF activity. We demonstrate the therapeutic efficacy of UR-12746 when administered orally at doses of 50 and 100 mg/kg in acute and chronic stages of the TNBS model of IBD^[92]. The intestinal anti-inflammatory effect of UR-12746 was associated with a decrease in leukocyte infiltration in the colon mucosa and with a reduction in myeloperoxidase activity. This effect was higher than that seen with sulphasalazine, when assayed at the same doses and in the same experimental conditions. This result suggests that the intestinal anti-inflammatory activity of UR-12746 by inhibition of leukotriene B₄ synthesis in the inflamed colon, improvement of the altered colon oxidative status, and reduction of colon IL-1 β production. Treatment with UR-12746 was able to ameliorate the altered colon oxidative status by significantly increasing glutathione content and by reducing the colon malonyldialdehyde levels^[92].

Future in therapy: Modulation of the immune response and biologic therapies

Advances in the understanding of IBD pathogenesis have allowed the development of novel therapies, which at least

theoretically represent a more specific management of the disease with fewer side effects. The real future in therapy should be to develop an approach to prevention of the initiation and perpetuation of the inflammatory status before tissue damage success, involving the induction of tolerance, commensal flora, generation of regulatory T cells and gene transfer, among others. Between these therapeutics factors, the immunosuppressors like calcineurin inhibitor (tacrolimus and cyclosporine), that suppresses pro-inflammatory cytokine production and T-cell activation, have been used to treat inflammatory bowel disease, especially in refractory ulcerative colitis, and to treat an extra-intestinal manifestation of IBD^[93]. Understanding the role of cytokines has been an important advance in IBD therapies, along with the monoclonal antibody technology, which made possible the targeted inhibition of specific disease related cytokines. Also, it has been possible to give inhibitory cytokines as therapeutic agents^[94]. The blockage of proinflammatory cytokine TNF- α (anti-TNF- α monoclonal antibody) serves as a model for development of new therapy^[94]. In this sense, the use of infliximab inhibits the bioactivity of TNF- α by directly binding to the cytokine and also modulates the function of TNF- α -producing cells^[95]. Many case reports have been published on the use of infliximab for treating patients with extraintestinal manifestations of IBD^[96].

Other potential modes of therapeutic actions in IBD include the induction of the anti-inflammatory cytokines IL-10 or TGF- β *via* retrograde signalling or induction of a certain subset of regulatory T cells^[97]. The IL-23 pathway has been a target of antibody blockade^[40]. Further research is needed to know how the *IL-23R* gene and the IL-23/IL-17-mediated inflammatory axis contribute to disease susceptibility and will lead to therapeutic interventions.

The process of T-cell activation, by enhancing regulatory T cells as opposed to inhibiting effector T cells could be considered to be effective for the treatment of IBD. Various populations of T cells exert a downregulatory effect on immune responses, including Tr1 cells (IL-10 secretion), Th3 cells (TGF- β) and CD4+CD25+ T regulatory cells, in which inhibition is through direct cell contact. The earliest work of this therapy may involve the selection and engineering of T cells delivering IL-10^[98].

Lymphocyte-endothelial interactions mediated by adhesion molecules are important in leukocyte migration and recruitment to sites of inflammation in IBD, a selective inhibition of these adhesion molecules offers many potential targets for specific intervention against inflammation^[99] to treat CD. In the next years, the role of anti-CD3 drugs (visilizumab), which induces apoptosis in activated T cells, the epithelial repair, and autologous hematopoietic stem cell transplants^[100] will be established.

Ultimately, the future therapy for IBD should be individualized and directed at induction of remission over a long period of time with the avoidance of important side-effects and maintaining the patient's quality of life.

CONCLUSION

Relevant advances in the understanding of the molecular

pathogenesis of IBD have been made, discovering susceptibility genes, identification of environmental factors implicated, and dysregulation of immunity in disease pathogenesis. Although the precise mechanisms underlying the development of IBD are not known, sufficient data have been collected to suggest interplay between genetic predispositions, accompanied by the importance of epithelial barrier function, and innate and adaptive immunity. Current therapies generally involve combinations of pharmacologic agents such as aminosalicylates, azathioprine, steroids, with dietary manipulation. Newer agents including monoclonal antibodies targeted to specific proinflammatory cytokines, to adhesion molecules, and the induction of anti-inflammatory cytokines and T-cell activation, have emerged and provided clinical benefit in the treatment and relapse of IBD.

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Role of endoscopy in the management of acute diverticular bleeding

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Abstract

Colonic diverticulosis is one of the most common causes of lower gastrointestinal bleeding. Endoscopy is not only a useful diagnostic tool for localizing the bleeding site, but also a therapeutic modality for its management. To date, haemostatic methods have included adrenaline injection, mechanical clipping, thermal and electrical coagulation or combinations of them. The results of all published data are herein reviewed.

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Forty percent of lower gastrointestinal bleeding (LGB) is due to colonic diverticulae. Inversely, bleeding complicates only 5% of all cases of colonic diverticulosis^[1]. In most of cases, hemorrhage ceases spontaneously; however, in 20% it persists, thus imposing an emergency treatment^[2].

Endoscopic hemostasis is a proven therapy in some indications of LGB, such as in the post-polypectomy bleeding and the post-radiation rectitis^[3,4]. Nevertheless, any attempt of endoscopic treatment presupposes either a certain diagnosis or a strong presumption of the bleeding

lesion, which may not always be evident. Since the end of 1980's, the benefit of an emergency colonoscopy in this setting has been investigated, but yielded contradictory conclusions (Table 1). After adequate bowel preparation urgent colonoscopy is incomplete in 0%-45% of cases and the risk of complications does not exceed 11%. The method allows positive diagnosis in approximately two thirds of cases and hemostasis in one third and may result in a shorter duration of hospitalization^[5-13]. The timing of colonoscopy has little or no impact on the diagnostic yield, but when it is performed early (within 24 h following admission) it seems associated with a better clinical outcome^[12-14].

The criteria proposed by Zuckerman for attributing LGB to diverticulosis are inspired by the Forrest classification of bleeding gastro duodenal ulcers^[15]. They include typical endoscopic stigmata such as active bleeding, visible vessel and adherent clot and presumptive findings, such as presence of fresh blood within one or more bowel segments and diverticular erosions. Using these criteria, a lower bleeding could undoubtedly be attributed to diverticulosis in only 20% of cases. In all other cases, the diverticular origin of bleeding is either presumed indirectly by the absence of any other lesion or considered as incidental^[16].

Endoscopic treatment aims to stop active bleeding, reduce the risk of recurrence, diminish transfusion needs and avoid surgery. To date, haemostatic methods have included adrenaline injection, mechanical clipping, thermal and electrical coagulation or associations of them. All of them are of similar efficacy, but mechanical clipping also offers the theoretical advantage of marking the bleeding site, which might be useful in case of relapse^[17]. Beyond these traditional techniques, Farrell, based on the previous work of Witte *et al*, has reported 4 cases of diverticular bleeding treated by elastic band ligation and suggested that this might be a promising method not only for the hemostasis, but for diverticular reversion as well^[18,19]. Concerning APC, despite its excellent results in vascular malformations, it has never been tested in the setting of diverticular hemorrhage, to our knowledge.

Apart of three original studies including only one prospective trial, all other published data are limited to some case reports. In the first study, Jensen *et al* compared 10 endoscopically managed patients with certain diverticular bleeding to 17 non-treated historical controls. Endoscopic treatment included diluted adrenaline injections in cases of active bleeding, bipolar coagulation in cases of visible vessel and association of

Table 1 Summarized data of the largest series evaluating the efficacy of endoscopy in the diagnosis and the management of lower gastrointestinal bleeding

Author	Publication year	Number of patients	Incomplete colonoscopy (%)	Positive diagnosis (%)	Endoscopic hemostasis (%)	Complications (%)
Jensen ^[5]	1988	80	0	74	39	5
Richter ^[6]	1995	78	11.5	90	13	NS
Geller ^[7]	1997	524	2	42	17	0.5
Kok ^[8]	1998	190	16	78	5	0
Prakash ^[9]	1998	30	0	60	6	0
Chaudhry ^[10]	1998	85	2	97	31	3.5
Ohyama ^[11]	2000	345	45	89	14	11
Schmulewitz ^[12]	2003	415	NS	89	10	0.002
Strate ^[13]	2003	144	5	90	10	NS

NS: Not stated.

Table 2 Summarized data of all studies evaluating the efficacy of therapeutic endoscopy in the management of diverticular bleeding

Author	Publication year	Type of study	n	Therapy	Early recurrence	Complications	Follow-up period (mo)
Johnston ^[21]	1986	Case Report	4	Coagulation	0	0	NS
Bertoni ^[22]	1990	Case Report	1	ADR	0	0	NS
Kim ^[23]	1993	Case Report	1	ADR	0	0	NS
Savides ^[24]	1994	Case Report	3	Coagulation	0	0	NS
Foutch ^[25]	1996	Case Report	4	Coagulation	1	0	NS
Ramirez ^[26]	1996	Case Report	4	ADR	0	0	NS
Hokama ^[27]	1997	Case Report	3	Clips	0	0	NS
Prakash ^[28]	1999	Case Report	3	Coagulation	0	0	NS
Jensen ^[16]	2000	Prospective	10	ADR, Coagulation, ADR + Coagulation	0	0	NS
Ohyama ^[11]	2000	Retrospective	6	ADR ± Coagulation ± Clips	0	0	NS
Bloomfeld ^[20]	2001	Retrospective	13	ADR ± Coagulation	5	0	35
Smoot ^[14]	2003	Retrospective	7	ADR (4), Clips (2), Thermocoagulation (1)	0	0	NS
Cuillier ^[29]	2003	Case Report	2	ADR	0	0	3-18
Simpson ^[30]	2004	Case Report	2	ADR + Clips	0	0	4-30
Total			63		6 (9.5%)	0 (0%)	

NS: Not stated.

both methods in cases of an adherent clot. None of the treated patients relapsed during a 30 mo observation period, whereas 9 of the 17 controls (52%) presented a recurrence within 3 years and 6 of them (35%) failed to avoid surgery^[16]. In the study of Bloomfeld, although the haemostatic methodology was similar to the previous study, results were less encouraging. Of 13 patients with active diverticular bleeding endoscopically treated, 5 and 3 presented an early or a late recurrence (up to 35 mo), respectively, thus a success rate of 46%^[20]. Finally, in the most recent study of Smoot *et al*, endoscopic haemostasis carried out in 7 patients actively bleeding, was proven both effective and free of complications^[14]. The methodology and the results of all relevant studies and reports are presented in Table 2. Summarizing the above data, one could say that out of 63 patients endoscopically managed, 6 (9.5%) presented an early relapse of bleeding and none a method-related complication. However, the long-term efficacy of endoscopic therapies is not demonstrated and randomized trials including larger number of patients are needed to make safe conclusions.

Thus, based on the above data one can conclude that in cases of lower GI bleeding, urgent colonoscopy after an adequate bowel preparation should be attempted. Endoscopic hemostasis might help patients to avoid at

least emergency surgery, if a diverticular origin seems certain or highly probable.

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TOPIC HIGHLIGHT

Peter Draganov, Dr, Series Editor

Endoscopic mucosal resection in the upper gastrointestinal tract

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Abstract

Endoscopic mucosal resection (EMR) is a technique used to locally excise lesions confined to the mucosa. Its main role is the treatment of advanced dysplasia and early gastrointestinal cancers. EMR was originally described as a therapy for early gastric cancer. Recently its use has expanded as a therapeutic option for ampullary masses, colorectal cancer, and large colorectal polyps. In the Western world, the predominant indication for EMR in the upper gastrointestinal tract is the staging and treatment of advanced dysplasia and early neoplasia in Barrett's esophagus. This review will describe the basis, indications, techniques, and complications of EMR, and its role in the management of Barrett's esophagus.

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Key words: Endoscopic mucosal resection; Mucosal resection; Barrett's esophagus; Barrett's dysplasia; Therapeutic endoscopy

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INTRODUCTION

Endoscopic mucosal resection (EMR) is a technique used

to locally excise lesions confined to the mucosa. Its main role in therapeutic endoscopy is the treatment of advanced dysplasia and early gastrointestinal (GI) cancers^[1-4]. Tada *et al* published the first case report of EMR in 1984 as a treatment option for early-stage gastric carcinoma^[5]. Since then, EMR has been used diagnostically and therapeutically in both the upper and lower GI tracts, and has significantly lower morbidity and mortality compared to surgical management^[6].

EMR is most commonly used to locally treat early gastric and esophageal dysplasia/cancer. However, there are several reports describing EMR as a therapeutic option for ampullary masses, colorectal cancer, and large colorectal polyps^[7-10]. In the Western world, the predominant indication for EMR in the upper GI tract is the staging and treatment of early neoplasia in Barrett's esophagus. This review will describe the basis, indications, techniques, and complications of EMR, and its role in the management of Barrett's esophagus.

BASIS OF EMR

The GI wall is comprised of 5 layers: mucosa, deep mucosa, submucosa, muscularis propria, and serosa or adventitia. The two major components are the mucosal and the muscular layer. The submucosa consists of loose connective tissue that attaches the above layers together. The mucosal and muscular layers can be separated from each other by injection of fluid into the submucosal layer. This allows for safe resection of mucosal lesions without causing damage of the deeper muscle layer^[11].

EMR has become standard practice because of its following distinct advantages: (1) advanced dysplasia and most early neoplastic lesions are free of lymph-node metastases, and can be treated with curative intent simply by local resection^[12]; (2) EMR provides tissue specimen for histology and staging^[13]; (3) EMR is minimally invasive and carries lower morbidity and mortality compared to traditional surgical approaches^[14]; and (4) surgery can be performed after EMR if advanced neoplasia or incomplete resection is detected on histologic evaluation of the EMR specimen.

EMR also has some disadvantages: (1) EMR is labor intense, time consuming, and requires advanced endoscopic skills; (2) larger lesions can only be resected in piecemeal fashion which precludes evaluation for completeness of the resection at the lateral margins; (3)

there is uncertainty regarding the long term outcome of patients treated with EMR due to the lack of randomized trials directly comparing EMR with surgery; and (4) EMR is poorly reimbursed in the US.

TECHNIQUES OF EMR

Once a mucosal lesion is identified, it is helpful to perform chromoendoscopy \pm endoscopic ultrasound to further define the size and borders of the lesion, and determine its depth of invasion^[15-17]. Both chromoendoscopy and narrow band imaging can help improve detection of dysplastic lesions, and further delineate the borders of the lesion. Additionally, computer tomography (CT) imaging can help determine the size of the lesion and rule out distant metastatic disease prior to proceeding with EUS. Although it can be challenging to determine intramucosal from submucosal neoplasias, the risk of pre-existing lymph node metastases must be discussed with the patient prior to pursuing EMR. Ultimately, histopathologic evaluation of the EMR specimen is the most important predictor of lymph node metastasis. Surgical management of early esophageal/gastric malignancies should be based on the histological analysis of the EMR specimen (i.e. depth of invasion) as well as each patient's surgical morbidity and mortality risks.

Several EMR techniques have been described in the literature. Multiple EMR techniques are available: (1) strip-off biopsy; (2) "inject, lift, and cut" method; (3) the "cup and suction" or EMR-cap technique; and lastly (4) EMR with band ligation^[18]. Prior to pursuing any of the above methods, it is recommended that the periphery of the lesion be marked with either a needle knife, electrocautery, or argon plasma coagulator (APC). This allows for distinct identification of the borders of the lesion that is being excised. The absence of all markings assists in determining if the resection is complete.

Depending on the EMR technique used, lift injection may be required. No standardization of the type of injection solution exists. Various injectates that have been used include normal saline, normal saline plus epinephrine solution, 50% dextrose in normal saline, 10% glycerine/5% fructose in normal saline, hyaluronic acid, and a mixture of methylene blue and normal saline^[18,19]. Injection of these various solutions can help lift the mucosa from the submucosa, and theoretically decrease the risk of perforation and reduce the risk of hemorrhage^[20]. An additional advantage of injecting prior to EMR is identification of lesions that do not successfully lift, which generally suggests involvement of the submucosal layer, and thus are not candidates for resection^[15].

Multiple electrosurgical currents are used during EMR, including blend, cut, and coagulation settings, depending primarily on operator preference. The electrosurgical setting most commonly used in the esophagus at Leeds is the ERBE "endo-cut mode" with a power setting of 45 watts^[21]. In a recent editorial by Seewald *et al.*, a pure coagulation current with the Erbotom at a setting 3- and 60-W output was used for electrosurgical resection^[22].

(1) The strip biopsy is the least complex EMR technique, but is often limited to polypoid or nodular

lesions only^[23]. It is similar to standard polypectomy. Injection into the submucosa is not done. A diathermy snare is tightened around a lesion, which is subsequently removed with the application of an electrical cutting current. The strip biopsy technique can be applied to flat lesions by using either a barbed snare or ultra stiff snare^[24].

(2) The "inject, lift, and cut" method is similar to the strip-off method. A two channel upper endoscope is needed. Prior to snaring the lesion, a submucosal injection is used to effectively lift the mucosa from the submucosa, thereby potentially reducing the risk of perforating the muscular layer. The lesion is then lifted by forceps and situated into a snare (*via* the second channel of a dual-channel endoscope), such that the lesion is resected at the base by applying electrocautery^[20].

(3) The "cup and suction" or EMR-cap technique is the most frequently used method of EMR in the esophagus^[25]. This technique was first described by Inohue *et al* in 1993^[26]. This method requires a transparent plastic cap be attached to the distal tip of a single-channel endoscope. This is followed by injection of approximately 20 mL of lifting solution into the submucosa. A designated "duck bill" small-diameter snare is then placed within the rim of the transparent cap. Following this, the lesion is sucked into the cap as the snare is closed at the base of the lesion. Once suction has been released and it has been determined that the entire lesion is contained within the snare, the lesion is removed using electrocautery^[27].

The advantages of the EMR-cap technique are that a standard single-channel endoscope can be used, and only one endoscopy assistant is required. In addition, it appears that there is a lower risk of bleeding compared to the strip-off method^[21,25]. The disadvantage is that it may be difficult to ensure that the entire lesion has been aspirated into the cap, and occasionally, visualization of the lesion can be obscured by the cap itself. Furthermore, the snare tends to lose its shape after a single use and thus a new snare is usually required for each piece of tissue removed. This can quickly add to the cost of the procedure, particularly if a large surface area needs to be removed (e.g. long segment Barrett's esophagus).

(4) EMR with ligation is similar to the EMR-cap technique, in that suction of the lesion is required. However, unlike the EMR-cap technique, the lesion is suctioned into a ligation cylinder without prior submucosal injection. A rubber band is then deployed at the base to create a pseudopolyp. The pseudopolyp is subsequently removed at its base by tightening a snare just below the level of the rubber band^[28]. The standard band ligator can be used, but a designated ligator that fits the single channel therapeutic upper scope is available (Duette, Cook Medical, Winston-Salem, NC). The main disadvantage of this technique with the standard ligator is that the endoscope must be withdrawn to remove the ligation cylinder before reinsertion for resection^[29]. The Duette system, on the other hand, allows the passage of a snare *via* the therapeutic channel of the scope and multiple resections can be carried out sequentially without the need of removing the scope.

The EMR-cap and EMR with ligation techniques have been prospectively compared to each other in a single-

center study performed by May A *et al*. In this study, 50 EMR-cap resections were compared to 50 EMR with ligation resections of early stage esophageal cancer. No significant difference in the maximal diameter of the resection specimen area was noted between the two groups. In addition, only one minor episode of bleeding was seen in each group, with no severe complications in either group. Therefore, it was concluded that both techniques are similar in efficacy and safety^[30].

ENDOSCOPIC SUBMUCOSAL DISSECTION (ESD)

ESD, one of the more recently described techniques, has been developed to perform single en-block resections of large mucosal lesions^[31]. This technique involves the use of an electrocautery knife to dissect out mucosal lesions. Several knives have been developed for ESD, including triangle-tip knives, hook knives, insulation-tip knives, and flex knives^[32-34]. In ESD with use of a triangle-tip knife, the borders of a mucosal lesion are marked by electrocautery and then injection of an epinephrine-saline solution into the submucosa is performed. This is followed by marginal cutting circumferentially, *via* electrocautery, around the previous markings. At this point, a high-viscosity solution (such as hyaluronic acid) is injected to provide a longer period of mucosal lifting to allow for actual submucosal dissection. Dissection is carried out by electro cauterization using the tip of the triangle-tip knife to free the mucosal lesion from the submucosa. Once completed, the freed mucosa is removed^[27]. At present, the various knives used for ESD are not available in the US.

EMR COMPLICATIONS

As with any endoscopic procedure, complication rates are operator dependent and diminish with increased experience. The Japanese Society of Gastrointestinal Endoscopy calculated a complication rate of 0.5% based on all upper GI EMR's performed between 1993 to 1997^[35]. A lower overall complication rate of 0.17% was reported by Kaneko *et al* in 1995. The mortality rate calculated by Kaneko *et al* was 0.0001%^[36].

The risks of EMR include bleeding, pain, perforation, and stricture formation. Bleeding can occur at the time of the procedure, or be delayed for up to 12 h. Bleeding rarely occurs beyond 24 h after the procedure. Venous oozing is more common following esophageal EMR, whereas brisk bleeding is more common after gastric EMR of large and fundic lesions. Bleeding is most commonly treated endoscopically *via* electrocauterization, APC, or placement of metallic clips^[37-40].

Dull pain following EMR generally results from denudation of the mucosa and subsequent exposure to gastric acid. This pain can often be controlled by proton-pump inhibitors. Sudden sharp pain, especially during or at the completion of the procedure, should raise suspicion of a perforation^[41]. The risk of perforation following EMR of gastric lesions is 0.06%-5%^[42]. Upper GI tract perforation can be managed conservatively with the combination of

clipping, nasogastric tube suction, and broad-spectrum antibiotics^[43].

Patients are at an increased risk of developing strictures if circumferential resections of the esophagus or gastric pylorus are performed^[29,44,45]. These strictures are often responsive to dilation. To date, animal studies have not identified that prophylactic balloon dilation, esophageal stenting, or deep mural steroid injections prevent the formation of strictures^[44].

INDICATIONS FOR EMR IN THE UPPER GI TRACT

Gastric malignancy

Per the Japanese literature, the indications for EMR in resection of superficial gastric cancers applies to well- or moderately differentiated adenocarcinoma and/or papillary carcinoma. Gastric cancers that penetrate the submucosa are at increased risk of lymph node metastases. Gastric cancer confined to the mucosa has a 0%-5% risk of lymph node metastases, compared to 10%-20% risk if the cancer involves the submucosa^[46-49]. Thus, gastric lesions must meet the following criteria to be candidates for EMR: confined to the mucosa, < 2 cm for elevated lesions, < 1 cm for flat or depressed lesions, cannot be associated with an ulcer or ulcer scar, and cannot have venous or lymphatic involvement^[50].

Esophageal squamous cell carcinoma

Currently, EMR is generally indicated for superficial well- or moderately differentiated squamous cell carcinoma without venous or lymphatic involvement that is limited to the lamina propria. This is based on a 0% risk of metastasis when the neoplasia is limited to the epithelium, compared to the 12% and 26% risk of metastasis when the neoplasia involves the muscularis mucosa and submucosa, respectively^[51]. There is no consensus on the maximum size of the lesion that can be resected. EMR is not recommended for circumferential lesions secondary to the risk of subsequent stricture formation^[15].

High-grade dysplasia and early adenocarcinoma in Barrett's esophagus (BE)

BE is associated with a 30-fold increased risk of esophageal adenocarcinoma, which remains one of most rapidly rising cancers in the Western world^[52-54]. Although EMR has a clear role in squamous cell carcinoma, its role in BE is not clearly defined, although studies have determined that EMR is effective in removing visible lesions in BE^[24,30,55]. In fact, current evidence suggests that EMR of high-grade dysplasia (HGD) and early cancer (EC) has similar success rates as surgical treatment^[56-60]. According to Ell *et al*, the indications for EMR in the setting of Barrett's neoplasia include the following: lesions limited to the mucosa that are macroscopically flat, tumor size between 20-30 mm, and good to moderate differentiation on histology^[56]. Additionally, research suggests that EMR has better diagnostic reproducibility compared to mucosal biopsies alone, implying a possible role in routine surveillance^[61]. Certainly, the cost-effectiveness and availability of EMR

would need to be considered prior to pursuing EMR as a primary tool in the surveillance of BE.

In one of the largest studies evaluating the efficacy of EMR for treatment of HGD and EC, 97% complete remission was achieved in resection of “low-risk” lesions and 59% complete remission in resection of “high-risk” lesions. “Low-risk” lesions were defined as macroscopic lesions measuring up to 20 mm and limited to the mucosa. However, at an average 12 mo follow-up period, the combined recurrence and metachronous cancer rate was 14%^[56]. In an Italian study, EMR was found to be an effective method of treating HGD and intramucosal cancer in 34 patients that did not have submucosal involvement, with all patients remaining in remission at a median follow-up of 34.9 mo. In addition, EMR changed the pretreatment diagnosis in 25.6% of the studied patients^[62].

Recurrence of neoplastic disease after EMR is a potential limitation^[56,59]. Therefore, circumferential resection, in which the targeted dysplastic lesion and the surrounding Barrett’s mucosa are removed, has also been studied^[63]. In two separate studies from 2003 and 2006, no recurrent or metachronous lesions were reported. This is in stark contrast to an 11% recurrence rate with circumferential resections at a mean follow-up of 18 mo in a study by Giovannini *et al.*^[24,29,55].

In addition to EMR, multiple ablative techniques have been evaluated in the management of HGD and EC in BE. These include photodynamic therapy (PDT), argon beam coagulation therapy, lasers, radiofrequency ablation, and yttrium-aluminum-garnet laser therapy^[64-68]. In fact, a recently-published multicenter, randomized controlled trial has shown that photodynamic therapy with Photofrin is superior than omeprazole alone in eliminating HGD at 5 years follow-up (77% *vs* 39%, respectively)^[69]. To improve eradication of neoplastic tissue and decrease recurrence rates, the combined use of EMR with ablative techniques have been described^[59,70]. Theoretically, recurrence rates are expected to be much lower with combined modalities, because both visible and non-visible lesions would be eradicated.

Combined modality has been evaluated by the Wiesbaden group in two separate studies. In the first study, 28 patients underwent EMR, 13 underwent PDT, 3 underwent APC, and 6 patients received a combination of these therapies for the treatment of HGD or EC. Metachronous or recurrent lesions were seen in 23% of the patients at a mean follow-up period of 34 ± 10 mo. Amongst the patients treated with EMR alone, 6/28 patients (21.4%) developed metachronous or recurrent lesions, compared to 1/6 patients (16.6%) treated with combined modalities^[59]. In a follow-up study of a total of 115 patients (EMR = 70, PDT = 32, APC = 3, EMR + PDT = 10) undergoing endoscopic treatment for HGD or EC, there was a 31% rate of metachronous or recurrent lesions over an average follow-up time of 34 ± 10 mo. Individually, the metachronous or recurrence rate was 30% (21/70) in the EMR group *vs* 37.5% in the EMR + PDT group^[70]. It is important to note that the number of patients treated with the combined modality is markedly less than that treated with EMR alone.

CONCLUSION

Since its introduction as a potential treatment option of GI mucosal cancers in 1984, the indications for EMR are continuing to expand. Today, EMR has become an integral part of the therapeutic endoscopy armamentarium. Although there are no specific guidelines for EMR as a treatment option for HGD or early cancer in Barrett’s esophagus, the literature indicates that EMR is similar to surgery in efficacy, but has less morbidity and mortality. As newer techniques of EMR, including circumferential mucosectomy, are developed, the potential of reducing recurrence and metachronous rates are inviting.

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GASTRIC CANCER

Relationship between cell adhesion molecules expression and the biological behavior of gastric carcinoma

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Abstract

AIM: To evaluate the relationship between the expression of cell adhesion molecules (CAMs) and the biological behavior of gastric carcinoma.

METHODS: Expression of syndecan-1, E-cadherin and integrin $\beta 3$ were evaluated by immunohistochemical study in a total of 118 gastric carcinomas and 20 non-tumor gastric mucosas.

RESULTS: The expressions of syndecan-1 and E-cadherin were significantly lower in gastric carcinoma compared to non-tumor gastric mucosa, and the low expression rates were positively correlated to the tumor invasion depth, vessel invasion, lymph node metastasis and distant metastasis ($P < 0.01$ in all cases). However, the expression of integrin $\beta 3$ was significantly higher in gastric carcinoma compared to non-tumor gastric mucosa, and the high expression rates were positively correlated to the tumor invasion depth, vessel invasion, lymph node metastasis and distant metastasis ($P < 0.01$ in all cases). In addition, the three protein expressions were correlated to the tumor growth pattern ($P < 0.01$, $P < 0.01$, and $P < 0.05$ respectively), but not correlated to tumor differentiation ($P > 0.05$, $P > 0.05$ and $P > 0.05$ respectively). Positive correlation was observed between the expressions of syndecan-1 and E-cadherin, but they which were negatively correlated to the expression of integrin $\beta 3$ ($P < 0.01$ in all cases). Univariate

analysis demonstrated that the mean survival time and 5-year survival rate were lower in the cases with low expressions of syndecan-1 and E-cadherin and high expression of integrin $\beta 3$ ($P < 0.01$, in all cases). COX multivariate analysis showed that the expression level of syndecan-1 could be an independent prognostic index of gastric carcinoma ($P < 0.01$), whereas E-cadherin and integrin $\beta 3$ could not be independent indexes ($P > 0.05$, $P > 0.05$ respectively).

CONCLUSION: The low expression of syndecan-1 and E-cadherin and the high expression of integrin $\beta 3$ are significantly correlated with the invasion and metastasis of gastric carcinoma, and they are highly correlated with each other. Therefore they may serve as important prognostic markers of gastric carcinoma.

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Key words: Cell adhesion molecules; Gastric Carcinoma; Invasion; Metastasis; Prognosis

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INTRODUCTION

Gastric carcinoma is the most common malignant tumor of the digestive system. Two of the most important causes for the high mortality are invasion and metastasis. However, the mechanism of invasion and metastasis of gastric carcinoma is still not definitely clear at present^[1]. Cell adhesion is one of the important steps in metastasis. Syndecan-1, E-cadherin and integrin $\beta 3$ make up cell adhesion molecules (CAMs) and participate in the adhesion between the cell and the extracellular matrix^[2]. Syndecan-1 is a set of transmembrane heparitin sulfate glycoproteins (HSPGS), which is present at the surface of most epithelia cells and take part in the adhesion between the cell and the extracellular matrix. The expression of syndecan-1 was augmented during epithelial regeneration and rearrangement in the stomach and other tissues^[3,4]. E-cadherin is a calcium dependent intercellular

adhesion molecule and it is present in normal cells to maintain the normal structure of tissues which has special biological characters and is highly related to invasion and metastasis of cancer cells^[3]. Studies show that the reduction or absence of syndecan-1 and E-cadherin expressions could induce the growth, invasion and metastasis of tumors^[5]. Integrin is a heterodimer consisting of a group of α and β polypeptide chains which can be divided into three groups based on the difference of a common β chain ($\beta 1$, $\beta 2$ and $\beta 3$). By identifying the extracellular matrix such as laminin, fibronectin and immunoglobulin superfamily on the cell membrane (I-CAM for example) *etc.*, mediating adhesive reaction of cell-extracellular matrix and cell-cell and receiving and conducting cascade signals, Integrin regulates the survival, apoptosis, movement, proliferation and inflammatory reaction of cells^[6]. To our knowledge, the co-expressions of syndecan-1, E-cadherin and integrin $\beta 3$ in gastric carcinoma and their clinical significances have not been reported before. We thus studied the expressions of CAMs in gastric carcinoma patients to find out the relationship among three of them using the immunochemical S-P method and to explore the correlation in their expression and pathological indexes of gastric carcinoma and survival time.

MATERIALS AND METHODS

Specimen

One hundred and eighteen specimens were collected from gastric carcinoma patients (91 men, 27 women, average age 56.3 years, range 25-79 years). They received the radical operations in our hospital from October 1986 to November 2002. Follow-up period was up to 5 years and the survival period was calculated from the day of surgery to the end of the follow-up or to the day of death. The censored value was zero. Of 118 gastric carcinomas, 70 were highly or moderately differentiated, while 48 were poorly or undifferentiated. According to the tumor, lymph node, and metastasis (TNM) standard stadiation, 47 were in T1-T2 stages and 71 were in T3-T4 stages. Eighty nine cases had vascular invasion and 29 without. Eighty three had lymph node metastasis and 35 without; distant metastases of carcinoma were found in 55 cases, while no distant metastasis in 63 cases. Twenty gastric mucosa specimens were collected 5 cm away from the cutting edges of radical operation as normal controls, which were detected as non-tumor mucosa.

Main reagents

Mouse anti-human syndecan-1, E-cadherin and integrin $\beta 3$ were purchased from ZYMED Company. SP kit was purchased from Maixin Biotect Company, Fuzhou, China. Streptavidin peroxidase staining was performed according to the kit instruction. Normal gastric mucosa was used as a positive control and PBS was used to replace the first antibody as a negative control.

Immunohistochemistry

Immunohistochemistry was made according to the streptavidin peroxidase (SP) methods. The staining step followed the routine process. In order to examine the

specificity of immunostaining, both positive and negative controls were run at the same time in each experiment. The normal gastric mucous was used as the negative control.

Results evaluation

Based on the proportion of positively stained cells in the sections, the criteria of syndecan-1 and E-cadherin were set as follows^[7]: (1) if more than 90% of the tumor cells exhibited intense membranous staining similar to that of normal cells, the result was considered as positive (++); (2) if the staining intensity was demonstrably reduced relative to that of normal cells and/or the staining pattern was heterogeneous (10%-90% positive), the result was deemed to be weakly positive (+); (3) if its expression was completely lost or positive in less than 10% of cells, the result was defined as negative (-). In statistical analysis, \pm were classified as the low expression group and ++ were set as the high expression group. In addition, the criteria for integrin $\beta 3$ were classified into four grades^[8]. Briefly, -, no staining in fewer than 10% of tumor cells; +, weak staining in only 10%-50% of tumor cells; ++, moderate staining in 50%-75% of tumor cells; and +++, strong staining of more than 75% of tumor cells. The sections for integrin $\beta 3$ were judged as a high expression group when more than 50% of cancer cells (++ or +++) were stained; others were judged as a low expression group.

Statistical analysis

SPSS 11.5 software package was used in data processing. Non-parameter Spearman rank correlation analysis was used to determine the relationship between the expressions of syndecan-1, E-cadherin and integrin $\beta 3$ and the pathological indexes of the progression of gastric carcinoma. The survival rate was estimated by the Kaplan-Meier method and analyzed by the long-rank test. Fisher's exact test was used to differentiate the rates of different groups. Univariate analysis and Cox-multivariate analysis were used to analyze the effect of the pathologic parameters (differentiation level, invasion depth, vessel invasion and lymph node metastasis), the expression of syndecan-1, E-cadherin and integrin $\beta 3$ on the total survival.

RESULTS

The expressions of syndecan-1, E-cadherin and integrin $\beta 3$ in gastric carcinoma and non-tumor gastric mucosa

Of a total of 118 gastric carcinomas the following was found: syndecan-1 low expression 57.6% (68/118) (Figure 1A) and high expression 42.4% (50/118); E-cadherin low expression 57% (68/118) (Figure 2A) and high expression 42.4% (50/118); and integrin $\beta 3$ low expression 50% (59/118) and high expression 50% (59/118) (Figure 3A). However, of 20 non-tumor gastric mucosa, syndecan-1 high expression (Figure 1B) 90% (18/20) and low expression 10% (2/20); E-cadherin high expression 85% (7/20) (Figure 2B) and low expression 15% (3/20); and integrin $\beta 3$ high expression 15% (3/20) (Figure 3B) and low expression 85% (17/20). Compared to normal tissues, the gastric carcinoma tissues showed lower syndecan-1 expression ($\chi^2 = 15.5$, $P < 0.01$), lower E-cadherin expression rate and density with

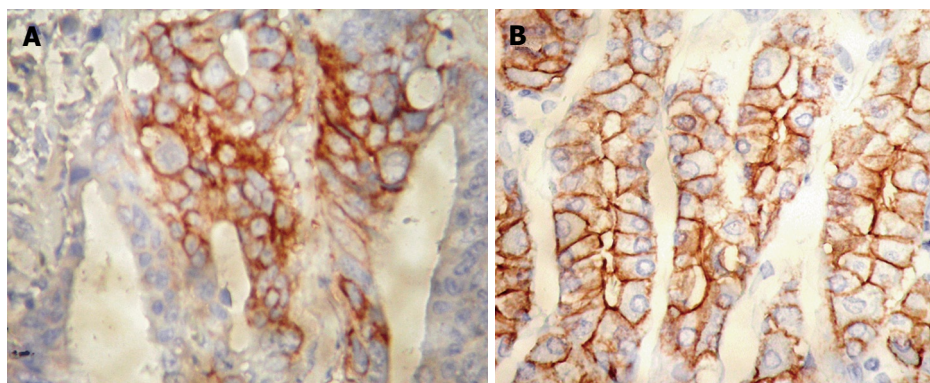


Figure 1 Syndecan-1 expression. **A:** Syndecan-1 was negatively (+) expressed in gastric carcinoma. *Sp method.* ($\times 250$); **B:** Syndecan-1 was highly expressed (++) in non-tumor gastric mucosa ($\times 250$).

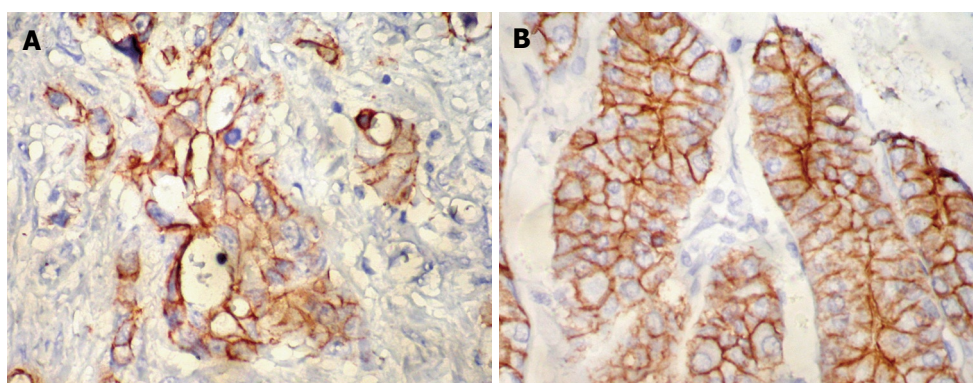


Figure 2 E-cadherin expressions. **A:** E-cadherin was negatively (+) expressed in gastric carcinoma (Magnification $\times 250$); **B:** E-cadherin was highly expressed (++) in non-tumor gastric mucosa ($\times 250$).

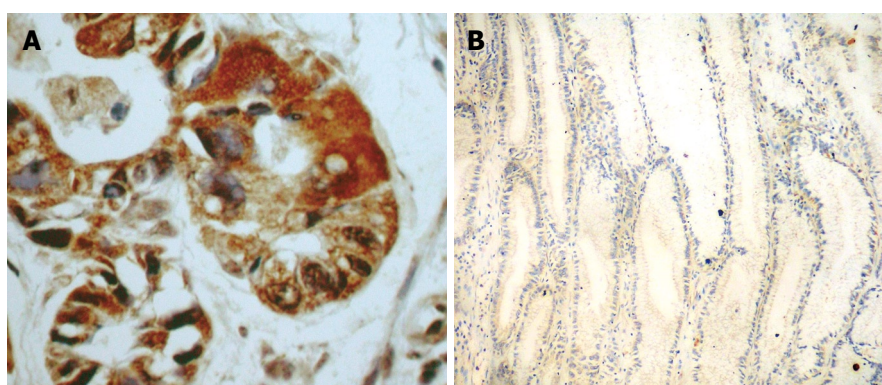


Figure 3 Integrin $\beta 3$ expression. **A:** Integrin $\beta 3$ was highly expressed (+++) in gastric carcinoma ($\times 250$); **B:** Integrin $\beta 3$ was negatively (+) expressed in non-tumor gastric mucosa ($\times 250$).

the increase of invasive depth of cancer cells ($\chi^2 = 12.4$, $P < 0.01$) and higher integrin $\beta 3$ expression ($\chi^2 = 8.5$, $P < 0.01$). There are significant differences in the expressions of these three proteins in gastric carcinoma and non-tumor gastric mucosa.

Relationship between the expression levels of syndecan-1, E-cadherin and integrin $\beta 3$ and the pathological indexes of progression of gastric carcinoma

The low expressions of syndecan-1 and E-cadherin were positively correlated with the gastric carcinoma growth mode ($\chi^2 = 12.47$, $P < 0.01$; $\chi^2 = 15.27$, $P < 0.01$), invasion depth ($\chi^2 = 32.95$, $P < 0.01$; $\chi^2 = 28.73$, $P < 0.01$), vessel invasion ($\chi^2 = 46.22$, $P < 0.01$; $\chi^2 = 40.52$, $P < 0.01$), lymph node metastasis ($\chi^2 = 43.49$, $P < 0.01$; $\chi^2 = 38.28$, $P < 0.01$) and distant metastasis ($\chi^2 = 63.30$, $P < 0.01$; $\chi^2 = 51.98$, $P < 0.01$). Additionally, the gastric carcinoma growing in invasive style had low expressions of syndecan-1 and

E-cadherin which were not correlated to the differentiation level of gastric carcinoma ($\chi^2 = 1.60$, $P > 0.05$). The high expression of integrin $\beta 3$ protein was positively correlated with gastric carcinoma growth modes ($\chi^2 = 5.83$, $P < 0.05$), invasion depth ($\chi^2 = 29.74$, $P < 0.01$), vessel invasion ($\chi^2 = 33.33$, $P < 0.01$), lymph node metastasis ($\chi^2 = 29.61$, $P < 0.01$) and distant metastasis ($\chi^2 = 41.72$, $P < 0.01$). The gastric carcinoma growing in invasive style had high expressions of integrin $\beta 3$ protein, which was not correlated to the differentiation level of gastric carcinoma ($\chi^2 = 0.14$, $P > 0.05$) (Table 1).

Relationship among the expressions of syndecan-1, E-cadherin and integrin $\beta 3$ in gastric carcinoma

There was significant positive correlation between the expression levels of syndecan-1 and E-cadherin. Both of them had negative correlation with integrin $\beta 3$ expression (Table 2).

Table 1 Correlation between expressions of syndecan-1, E-cadherin and integrin $\beta 3$ and pathological parameters in 118 gastric carcinoma patients

Variable	(n)	Syndecan-1 expression				E-cadherin expression				Integrin $\beta 3$ expression			
		Low	High	χ^2	P	Low	High	χ^2	P	Low	High	χ^2	P
Growth mode of tumors													
Invasion	67	48	19	12.47	0.000	49	18	15.27	0.000	27	40	5.836	0.016
Expansion	51	20	31			19	32			32	19		
Histologic differentiations													
Well/moderate	70	37	33	1.60	0.205	37	33	1.60	0.205	36	34	0.140	0.708
Poor	48	31	17			31	17			23	25		
Depth of invasion													
T1-T2	47	12	35	32.95	0.000	13	34	28.73	0.000	38	9	29.74	0.000
T3-T4	71	56	15			55	16			21	50		
Vascular invasion													
Negative	29	1	28	46.22	0.000	2	27	40.52	0.000	28	1	33.33	0.000
Positive	89	67	22			66	23			31	58		
Lymphatic metastasis													
Negative	35	4	31	43.49	0.000	5	30	38.28	0.000	31	4	29.61	0.000
Positive	83	64	19			63	20			28	55		
Distant metastasis													
Negative	63	15	48	63.30	0.000	17	46	51.98	0.000	49	14	41.72	0.000
Positive	55	53	2			51	4			10	45		

Table 2 Relationship among the expressions of syndecan-1, E-cadherin and integrin $\beta 3$ in gastric carcinoma

Groups	Syndecan-1			r	P	E-cadherin			r	P
	-	+	++			-	+	++		
Syndecan-1										
-	43					38	4	1		
+		25				11	12	2		
++			50			3	0	47		
E-cadherin				0.837	0					
-	38	11	3			52				
+	4	12	0				16			
++	1	2	47					50		
Integrin $\beta 3$				-0.792	0				-0.666	0
-	2	6	48			9	1	46		
+	1	1	1			2	0	1		
++	18	8	1			19	8	2		
+++	22	10	0			22	7	1		

Factors that affect the survival rate of gastric carcinoma

Univariate analysis showed that the patients with high syndecan-1 expression had a 5-year survival rate of 91.66%, while it was 12.8% in those with low syndecan-1 expression. There was significant difference between the rates ($\chi^2 = 53.13$, $P < 0.01$). The patients with high E-cadherin expression level had a 5-year-survival rate of 93.59%, which was significantly different from that of the patients with low E-cadherin expression with the rate of 12.8%. There was significant difference between the rates ($\chi^2 = 43.36$, $P < 0.01$). The patients with high integrin $\beta 3$ protein expression had a 5-year-survival rate of 13.85% and the patients with low integrin $\beta 3$ expression had the rate of 72.75%. There was significant difference between the rates ($\chi^2 = 35.11$, $P < 0.01$). Kaplan-Meier analysis indicated that the patients with low syndecan-1/E-cadherin protein expression level and high integrin $\beta 3$ expression level had poor prognosis (Table 3, Figure 4A-C).

COX-multivariate analysis showed that syndecan-1 expression could be used as a prognostic marker of gastric

Table 3 Relationship between the expressions of syndecan-1, E-cadherin and integrin $\beta 3$ and the prognosis of gastric carcinoma patients

Groups	n	Mean survival time (mo)	5-yr survival rate (%)	χ^2	P
Syndecan-1 expression					
Low expression	68	32.10 \pm 4.16	12.80	53.13	0
High expression	50	123.80 \pm 5.56	91.66		
E-cadherin expression					
Low expression	68	33.69 \pm 4.33	12.80	43.36	0
High expression	50	119.78 \pm 5.87	93.59		
Integrin $\beta 3$ expression					
Low expression	59	103.30 \pm 7.73	72.75	35.11	0
High expression	59	33.12 \pm 4.64	13.85		

cancer patients ($P < 0.01$). However, E-cadherin and integrin $\beta 3$ could not be used as independent prognosis markers ($P > 0.05$ and $P > 0.05$ respectively). (Syndecan-1: B = 3.447, SE = 0.988, Wald = 12.183, $P < 0.01$; E-cadherin: B = 0.019, SE = 0.686, Wald = 0.001, $P > 0.05$; integrin $\beta 3$: B = 0.098, SE = 0.364, Wald = 27.711, $P > 0.05$).

DISCUSSION

Gastric carcinoma is highly malignant and usually results in a poor prognosis. Although the achievement in early diagnosis and treatment of gastric cancer has improved the patients' outcome, it is still one of the leading causes of mortality in countries such as China and Japan. Currently, about 39% of gastric cancer cases occur in the Chinese population, ranking the leading cause of cancer-mortality in China, particularly in rural areas^[9]. The overall 5-year survival rate for patients who undergo curative surgical resection for gastric carcinoma ranges from 47% to 60.4%^[10]. The typical characteristics of malignant tumor are invasion and metastasis, which are the main cause for their lethality. Tumor progression is considered to be

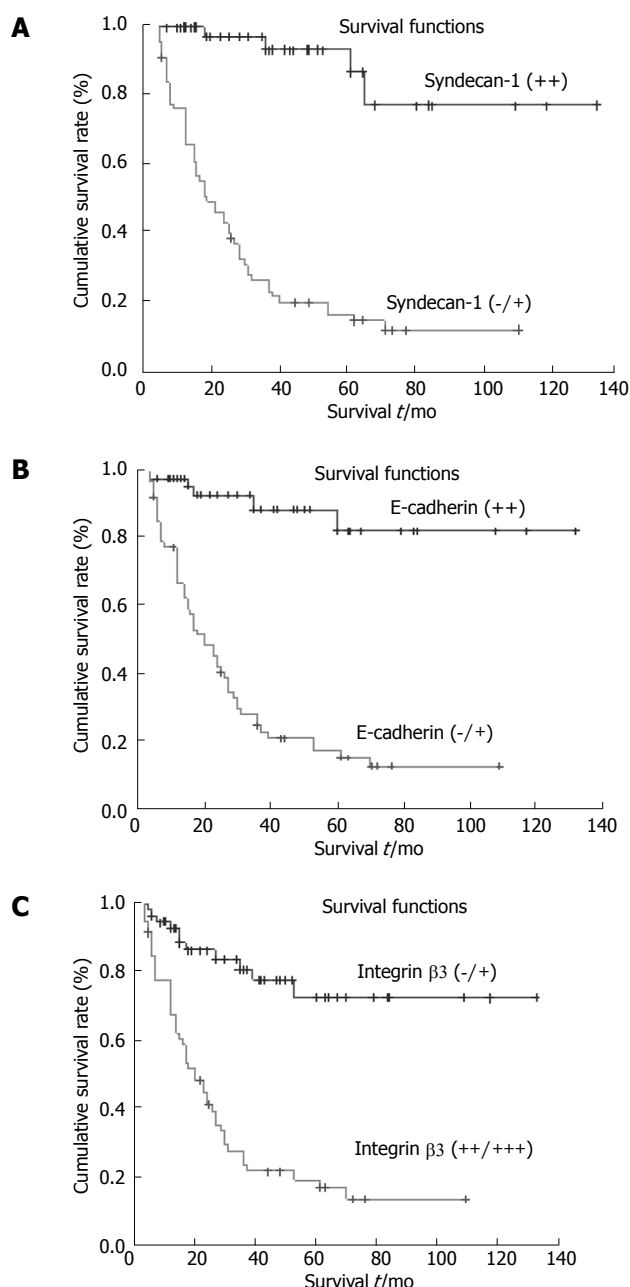


Figure 4 Correlation between syndecan-1, E-cadherin and integrin $\beta 3$ expressions and the survival time. **A:** Survival curves by the Kaplan-Meier method. Long-rank test revealing a significant difference between negative and positive expression of Syndecan-1 ($P < 0.05$); **B:** Survival curves by Kaplan-Meier method. Long-rank test revealing a significant difference between negative and positive expression of E-cadherin ($P < 0.05$); **C:** Survival curves by the Kaplan-Meier method. There was a significant difference in Long-rank test between positive and negative expression of integrin $\beta 3$ ($P < 0.05$).

dynamic, complex, and a multi-step process, where the essential steps are the breakdown of cell-cell adhesion and degradation of basement membrane (BM)^[11]. The cellular and molecular steps required for metastases of neoplastic cells are starting to be elucidated. The metastatic cascade starts with a downregulation of the epithelial bonds which enables tumor cells to leave epithelial structures, to invade the stroma, to enter the blood stream or the lymphatics, to extravasate, and to colonize the target organs. Interestingly, invasive growth and metastases often recapitulate embryonic development and are greatly influenced by

the stromal microenvironment^[12]. Thus, carcinoma cells interact bidirectionally with neighboring fibroblasts, endothelial cells, and lymphocytes through growth factors, chemokines, cell adhesion molecules, and extracellular matrix proteases.

Loss of cell adhesion may contribute to loss of contact inhibition of growth, which is an early step in the neoplastic process. It has been shown that various cell adhesion molecules expressed on carcinoma cells play crucial roles. However, the mechanisms of invasion and metastasis are still under investigation. Until now there is no satisfactory tumor marker for predicting its evolution.

Syndecan-1, E-cadherin and integrin $\beta 3$ make up CAMs together^[2] to participate in adhesion between cell and extracellular matrix. Experimental studies show that changes in cell-cell and cell-matrix adhesion are central to the conversion from premalignant lesions to early invasive carcinoma^[13].

Syndecan-1 (CD138) is a member of the transmembrane heparin sulfate proteoglycan (HSPG) family, taking part in and improving adhesion between cell and extracellular matrix^[14], improving cell proliferation, maintaining the differentiation phenotype of cells and inhibiting the growth of tumor cells^[15]. Wiksten *et al*^[16] reported that abatement or loss of syndecan-1 expression is highly correlated to the focal size, lymphatic metastasis, invasion depth, TNM stage and prognosis of gastric carcinoma. It is reported that the expression of syndecan-1 increases gradually from large intestine adenoma to carcinoma, then to invasive carcinoma^[17,18].

E-cadherin is a calcium dependent transmembrane glycoprotein and has the functions of mediating the adhesion of homogeneous cells among epithelia and of maintaining the integrity and polarity of tissue structures^[19]. The abatement or loss of E-cadherin expression may induce the decrease of adhesion among cells and thus make cancer cells disunite, grow invasively toward peripheral tissues and leave original focal to form metastasis once the necessary conditions are met^[20,21]. Documents indicate that in gastric carcinomas, the reduction in E-cadherin expression activation of E-cadherin gene varies from 17% to 92%^[22,23]. This phenomenon has been confirmed in many kinds of tumors such as lung cancer, breast cancer, large intestine cancer, liver cancer and gastric cancer^[24-27].

Integrins belong to the family of transmembrane glycoprotein hetero-dimer. They mediate adhesion of neighboring cells and participate in the growth and repair of cells and vascular proliferation *etc* as important receptors of extracellular matrix protein^[28]. Molecular biological studies on melanoma, colon and rectal cancer and other carcinomas in the past few years showed that the dissociation from or penetration through BM of tumor cells, caused by the adhesion of $\alpha 3\beta 1$, $\alpha 5\beta 1$, $\beta 3$ and other Integrins on tumor cell surface to extracellular matrix, is the initial step for the invasive growth and remote metastasis of malignant tumor. Moreover the high expression of integrin $\beta 3$ in malignant melanoma and malignant ovarian tumor cells is positively correlated to invasion and metastasis of cancer cells^[29].

Documented data shows that these three proteins, syndecan-1, E-cadherin and integrin $\beta 3$, cooperate with

each other for expressions and functions; moreover their expression levels are correlated with the progression of gastric carcinoma. Experimental studies show that syndecan-1 and E-cadherin are all present in epithelia and both can form immunoprecipitation with transcription regulatory factor β -cadherin and this indicates that they are materially and functionally correlated with each other^[30]. The expression of syndecan-1 and E-cadherin in gastric carcinoma is low and their expression levels are positively correlated^[31]. The abatement or loss of E-cadherin expression is involved in lymph node micro-metastasis of gastric carcinoma^[32]. Sun *et al.*^[33] proposed the following point of view based on different studies: the decrease of *in vitro* syndecan-1 expression inhibits E-cadherin expression, and/or lowers E-cadherin expression at the same time as the beginning of epithelium-stroma transformation and induces effective and timely epithelium-stroma transformation^[33].

Signal conduction mediated by syndecan-1 needs the cooperation of integrin $\beta 3$ ^[34]. Mammary glandular epithelia short of syndecan-1 show rearrangement of integrin $\beta 3$ and markedly low expression of E-cadherin at the same time^[35]. Wound healing theory indicates that E-cadherin activates the migration of integrin $\beta 3$ -transfected cells and constrains them to separate from wound margin. The study of Ohta *et al.* shows that over expression of the homeobox gene HOXD3 promotes the non-expression of E-cadherin and increased expression of integrin $\beta 3$, which plays an important role in the quick migration and isolation of tumor cells^[29]. Zhang *et al.*^[11] reported that E-cadherin loss in epithelial tumor progression was not only related to severing cell-cell adhesion but also associated with increased integrins expression, which induced cell-matrix adhesion of these cells^[13].

The results of this study indicate that the expressions of syndecan-1 and E-cadherin in gastric tumor tissues are low and their levels are significantly correlated. This suggests that syndecan-1 and E-cadherin play a positively cooperative role in the genesis and development of gastric carcinoma. They are negatively correlated to the level of integrin $\beta 3$ expression and this suggests that Integrins have different effects on the progression of gastric carcinoma. The functional consequence of enhanced cell-matrix adhesion is the initial attachment and retention of these cells at the epithelial-stromal interface, thus providing the appropriate microenvironmental conditions for incipient tumor cell invasion^[13].

Basing on the biological characters of syndecan-1, E-cadherin and Integrins, on relevant documents and on the results of this study, the correlation of CAMs with the progression of gastric carcinoma can be summarized as follows: (1) at the early stage of gastric carcinoma, tumor cells have low E-cadherin and syndecan-1 expression, adhesion between cells is weak and tumor cells are isolated from primary tumor, which is the initial step of invasion and metastasis of gastric carcinoma; (2) the decrease of the expressions of E-cadherin and syndecan-1 reduces adhesion between tumor cells and BM or extracellular matrix mediated by them and this is propitious to local growth and dispersion of tumor. At the same time, various hydrolytic enzymes will be released, after the adhesion of

cancer cells to BM or extracellular matrix, to degrade the BM or extracellular matrix which tumor cells adhere to. Thus cancer cells may enter into the blood circulation. Experimental studies show that Integrins are the main receptors for cells adhesion to extracellular matrix and syndecan-1 increases the combination of them; (3) the increase of Integrin expression, after that cancer cells enter the blood circulation, is in favor of adhesion of tumor cells to endothelia to induce invasive growth and remote metastasis of gastric carcinoma. In these processes, adhesion of tumor cells to endothelia and the BM under endothelia is the key process for the invasion and metastasis.

The pathological indexes and survival analysis results confirm that gastric cancer tissues with low syndecan-1 and E-cadherin protein expressions and high integrin $\beta 3$ protein expression have deeper invasion depth, higher occurrences of vascular invasion, lymph node metastasis and remote metastasis, shorter average survival time and lower 5-year survival rate, which is consistent with the study results of Wiksten *et al.*^[16], Trikh *et al.*^[36] and other researchers.

In conclusion, low expressions of syndecan-1 and E-cadherin protein and high expression of integrin $\beta 3$ protein are significantly correlated to invasion of gastric carcinoma. As intracellular adhesion molecular complexes, these three proteins are highly correlated with each other. Therefore, the results of co-examination of them can be important indexes for prognosis of gastric carcinoma.

COMMENTS

Background

Cell adhesion is one of the important steps in invasion and metastasis. Syndecan-1, E-cadherin and integrin $\beta 3$ make up intercellular adhesion molecules (CAMs) and participate in the adhesion between cell and extracellular matrix. One or two of them in gastric carcinoma has been reported before; however, in order to better understand the coordinated regulation of cell-cell and cell-matrix interactions during malignant transformation, we study the coexpression of E-cadherin, syndecan-1 and integrin $\beta 3$ by immunohistochemical study in gastric carcinomas.

Research frontiers

Recent investigations have suggested that frozen tissue-based molecular classifications effectively predict prognosis of gastric cancer, prognostic classification on formalin-fixed tissue is needed. Therefore additional markers are required in the prognosis of patients with gastric cancer.

Innovations and breakthroughs

In this article, we identified that syndecan-1, E-cadherin and integrins were highly correlated with each other as intracellular adhesion molecular complexes. We suggest that the results of co-examination can be important indexes for prognosis of gastric carcinoma.

Applications

The results from the study confirm the correlation between expressions of CAMs and gastric cancer. It suggests that the coexpression of them can be used to identify the prognosis of gastric carcinoma.

Terminology

CAMs are proteins located on the cell surface involved in the binding with other cells or with the extracellular matrix in the process called cell adhesion.

Peer review

This report analyzed that advanced gastric cancer including with/without distant

metastases. Prognostic factor of each stage of gastric cancer about CAMs should be analyzed and author should speculate other factors too.

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Long-term outcome of percutaneous ethanol injection therapy for minimum-sized hepatocellular carcinoma

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Abstract

AIM: To evaluate long-term follow-up of minimum-sized hepatocellular carcinoma (HCC) treated with percutaneous ethanol injection (PEI).

METHODS: PEI was applied to 42 lesions in 31 patients (23 male and eight female) with HCC < 15 mm in diameter, over the past 15 years.

RESULTS: Overall survival rate was 74.1% at 3 years, 49.9% at 5 years, 27.2% at 7 years and 14.5% at 10 years. These results are superior to, or at least the same as those for hepatic resection and radiofrequency ablation. Survival was affected only by liver function, but not by sex, age, etiology of Hepatitis B virus or Hepatitis C virus, α -fetoprotein levels, arterial and portal blood flow, histological characteristics, and tumor multiplicity or size. Patients in Child-Pugh class A and B had 5-, 7- and 10-years survival rates of 76.0%, 42.2% and 15.8%, and 17.1%, 8.6% and 0%, respectively ($P = 0.025$).

CONCLUSION: Treatment with PEI is best indicated for patients with HCC < 15 mm in Child-Pugh class A.

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Key words: Percutaneous ethanol injection; Interventional ablation; Ultrasound; Hepatocellular carcinoma; Prognosis

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INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the major malignancies worldwide^[1-4]. With recent advances in diagnostic imaging, particularly in ultrasound (US), an increasing number of small or early-stage HCCs have been detected. In patients with early-stage HCC, percutaneous ethanol injection (PEI) has been a second choice when surgical techniques have been precluded, although PEI has been used as a first-line treatment option in some centers in Italy and Japan. Over the past few years, several methods for thermal tumor destruction through localized heating or freezing, including radiofrequency ablation (RFA), laser ablation, microwave ablation, and cryoablation, have been developed and clinically tested. Among these, RFA has recently emerged as a real competitor to PEI.

At this point, there are no unequivocal data to back up percutaneous ablation as a replacement for resection as first-line treatment for patients with early-stage HCC. Therefore, whether percutaneous ablation replaces resection as first-line option for very early HCC will be resolved by launching a large international randomized controlled trial.

In this study, early-stage HCC (< 15 mm diameter) is referred to as minimum-sized HCC^[5]. The present study is a long-term follow-up of minimum-sized HCC treated with PEI.

MATERIALS AND METHODS

Subjects

We analyzed the clinical features of consecutive patients treated with PEI in the 1990s at Kobe Asahi Hospital. The diagnosis of HCC was made by imaging, including US and computed topography (CT) and confirmed by

tumor-targeted biopsies. Thirty-one patients with liver cirrhosis and HCC were treated with PEI as the first-line anticancer treatment. The characteristics of the patients are summarized in Table 1. The criteria for treatment with PEI were as follows: (1) Uninodular HCC ≤ 15 mm in diameter, or multinodular HCC lesions ≤ 15 mm in diameter (in one or both hepatic lobes); (2) absence of portal vein thrombosis and extra-hepatic metastases; (3) age < 75 years; (4) liver cirrhosis of Child-Pugh class A or B; and (5) prothrombin time ratio (normal/patient) $> 40\%$ and platelet count $> 40\,000/\mu\text{L}$. The number of tumorous nodules and portal vein patency were established by US and CT. Maximum tumor diameter was measured by US. The absence of extrahepatic metastases was ascertained by chest X-ray, and abdominal CT and US.

Liver cirrhosis was diagnosed histologically, radiologically or clinically. Serum hepatitis B surface antigen (HBsAg) was positive in two patients, and anti-hepatitis C virus antibody (anti-HCV) was positive in 29. Arterial blood flow was confirmed by CO₂ US-angiography or CT during arteriography (CTA); portal blood flow was confirmed by CT during arterial portography (CTAP) (Table 1). Surgery was contraindicated in most patients because of liver dysfunction, presence of lesions in locations that made hepatic resection inappropriate, advanced age, coexistence of another disease, or a combination of these factors. However, during the past few years, some patients, including possible candidates for surgery, were treated with PEI. Informed consent was obtained from all patients after the nature of the procedure had been fully explained.

The diameter of the tumors ranged between 8 and 15 (mean 11.9 ± 2.4) mm. The diagnosis of HCC was established by histological biopsy with the needle guided by sonography in 31 patients.

Treatment schedule and follow-up protocol

PEI^[6] was administered to each patient (3-6 sessions; once or twice weekly) by one or two injections of 95% sterile ethyl alcohol (1.6-73.9 mL, mean 17.6 ± 16.7 mL) delivered to each lesion with a multiple-side-hole 21-gauge needle (Et-hanoject, TSK, Tokyo, Japan), depending on the size of the lesion and the distribution of the injected ethanol within the tumor.

One month after the end of PEI treatment, the α -fetoprotein (AFP) level was measured, CT was repeated, and multiple percutaneous biopsies of the treated lesions were carried out to evaluate treatment outcome. Lesions appearing as hypoattenuated, non-enhanced areas on CT scans were diagnosed as necrotic, whereas enhanced areas were suspected of being persistent tumors. The biopsies under US guidance were carried out by placing the needle in the enhanced areas of the tumor. Histological samples were obtained from all of the patients.

The treatment was terminated and the patients entered the follow-up protocol in the absence of residual tumors, confirmed by CT and biopsies, and of suspected persistent tumors, confirmed by AFP levels. Such patients were given additional PEIs targeted in areas where viable tumors had previously been observed, and were examined again by CT

Table 1 Characteristics of 31 patients with liver cirrhosis and small HCC

Parameter	
Sex (male:female)	23:8
Age (yr) (mean \pm SD)	63.8 \pm 8.9
Cause of cirrhosis	
HCV	29
Non-HCV	2
Liver dysfunction	
Child-Pugh A	17
Child-Pugh B	14
AFP level ($\mu\text{g/L}$)	
≤ 20	15
> 20	16
Tumor multiplicity	
Uninodular	21
Multinodular	10
Tumor diameter (mm) (mean \pm SD)	11.9 \pm 2.4
≤ 10	12
11-15	19
Histological characteristics	
Well-differentiated	18
Well to moderately differentiated	13
Arterial blood flow	
Positive	9
Negative	13
Portal blood flow	
Positive	12
Negative	9

and biopsies after 1 mo. The follow-up protocol included clinical assessment, measurement of hepatic functional serum indexes and AFP levels, and US examinations conducted at 3-mo intervals. The duration of the follow-up was calculated from the beginning of PEI and lasted for 2-167 (mean \pm SD, 57.5 ± 37.7) mo.

Statistical analysis

Student's *t* test and χ^2 test were used to identify differences in patient characteristics in the various subgroups with prognostic factors. The Kaplan-Meier method was used to analyze the factors associated with post-PEI survival of patients with HCC and distant intrahepatic recurrence of HCC, and the difference was determined by log rank test. Stepwise regression analysis was used to identify factors that affected the survival rate of post-PEI patients. $P < 0.05$ was considered to indicate a statistically significant difference.

RESULTS

All patients completed the planned treatment course. No major treatment-related complication had occurred by the end of the study. Overall survival rate was 74.1% at 3 years, 49.9% at 5 years, 27.2% at 7 years and 14.5% at 10 years. The longest survival period was 13 years 11 mo, and three patients lived longer than 10 years after PEI treatment. Up to December 2003, 23 post-PEI patients died: Four from cancer (17.4%), 15 from hepatic failure (65.2%) and four from other causes (17.4%). According to the Child-Pugh classification, two (20%) died from cancer and seven (70%) from hepatic failure (class A), and

Table 2 Cause of death of post-PEI patients with small HCC, according to Child-Pugh classification and number of treatment sessions

	Child-Pugh classification		Number of treatment sessions				
	A (n = 10)	B (n = 13)	1st	2nd	3rd	4th	5th
Cancer growth	2	2	0	3	0	0	0
Hepatic failure							
Cancer (+)	7	6	3	2	2	1	5
Cancer (-)	0	2					
Other	1	3	3	0	0	0	0

Table 3 Analysis of the prognostic value of patient- and tumor-related factors

Factor	n	Probability of survival (%)				P value
		3-yr	5-yr	7-yr	10-yr	
Sex						0.595
Male	23	73.7	59.5	29.7	11.1	
Female	8	62.5	33.3	0	0.0	
Age (yr)						0.475
≤ 65	18	77.4	59.5	29.8	9.9	
> 65	13	61.5	35.9	23.9	0.0	
Cause of cirrhosis						
HCV	29	72.2	46.2	23.1	15.4	
Non-HCV	2	-	-	-	-	
Liver dysfunction						0.025
Child-Pugh A	17	76.0	76.0	42.2	15.8	
Child-Pugh B	14	64.3	17.1	8.6	0.0	
AFP level (μg/L)						0.139
≤ 20	15	79.4	56.9	42.6	21.3	
> 20	16	62.5	37.5	18.7	0.0	
Tumor multiplicity						0.751
Uninodular	21	75.9	50.6	36.1	13.5	
Multinodular	10	60.0	37.5	12.5	12.5	
Tumor diameter (mm)						0.336
≤ 10	12	74.1	64.8	32.4	21.6	
11-15	19	68.4	40.9	24.6	12.2	
Histological characteristics						0.119
Well-differentiated	18	83.3	58.0	33.2	22.1	
Well to moderately differentiated	13	61.5	38.5	19.2	0.0	
Arterial blood flow						0.269
Positive	9	64.8	38.9	38.9	38.9	
Negative	13	61.5	30.8	10.2	0.0	
Portal blood flow						0.458
Positive	12	58.3	33.3	16.7	0.0	
Negative	9	64.8	38.9	38.9	0.0	

two (15.3%) from cancer and eight (61.5%) from hepatic failure (class B) (Table 2).

Prognostic factors

The influence of patient- and tumor-related factors on survival is shown in Table 3. Survival was affected by liver function, but not by sex, age, etiology of cirrhosis (HCV or non-HCV), AFP level, arterial blood flow, portal blood flow, histological characteristics, and tumor multiplicity or size. The final step of stepwise variable showed function was significantly associated with survival rate. Child-Pugh class A patients showed a higher survival rate than Child-Pugh class B ($P = 0.011$, Table 4). The 5-, 7- and 10-years

Table 4 Factors affecting survival of patients with HCC treated by PEI, determined by stepwise regression analysis

Factors	Hazard ratio	95% CI	P value
Child-Pugh class			
A	1		
B	5.89	1.50-23.15	0.011

Table 5 Characteristics of patients according to Child-Pugh class

Parameter	Class A (n = 17)	Class B (n = 14)	P value
Sex (male:female)	16:1	7:7	0.017
Age (yr) (mean ± SD)	61.2 ± 8.3	66.9 ± 8.9	NS
Cause of cirrhosis			
HCV	15	14	NS
Non- HCV	2	0	
AFP level			NS
≤ 20 μg/L	9	6	
> 20 μg/L	8	8	
Tumor multiplicity			NS
Uninodular	11	10	
Multinodular	6	4	
Tumor diameter (mm) (mean ± SD)			NS
≤ 10	7	5	
11-15	10	9	
Histological characteristics			NS
Well-differentiated	9	9	
Well to moderately differentiated	8	5	
Arterial blood flow			NS
Positive	5	4	
Negative	5	8	
Portal blood flow			NS
Positive	7	5	
Negative	4	5	

NS: Not significant.

survival rates of 76.0%, 42.2% and 15.8%, respectively, for patients in Child-Pugh class A were significantly higher than those for patients in Child-Pugh class B (17.1%, 8.6% and 0%, respectively) ($P = 0.025$, Figure 1). Patient characteristics by Child-Pugh classification showed that prognosis of class A and class B patients was affected by sex and not by any other patient- and tumor-related factors (Table 5). The two groups were similar with respect to the other patient- and tumor-related factors.

Lesions in intrahepatic areas other than the site treated by PEI were observed in 25 patients (81.8%), and cumulative recurrence rates at 1, 3, 5, 7 and 10 years after PEI were 47.0%, 73.4%, 87.7%, 87.7% and 87.7%, respectively (Figure 2). The frequency of initial recurrence according to segment was 32.0% in segments other than the initially treated segment (other segments), 32.0% in the same segment as the initially treated segments, and 28.0% in both the same and other segments. The tumor diameter of recurrent lesions was < 20 mm in 84.0% of cases, 21-30 mm in 8.0%, and no tumor was > 30 mm at detection (Table 6). The recurrent lesions that occurred in 25 post-PEI patients after initial treatment were managed as follows: PEI in 15

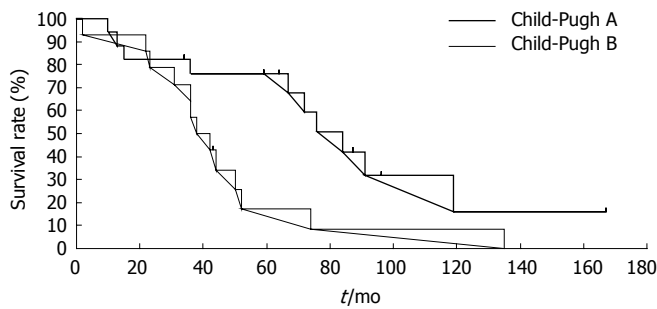


Figure 1 Survival of patients with HCC treated by PEI according to Child-Pugh classification.

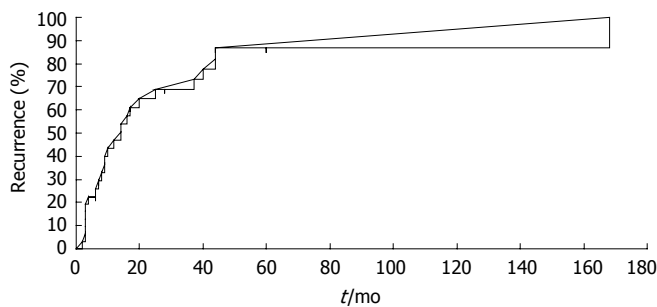


Figure 2 Recurrence of HCC treated by PEI at remote site from initial treated site.

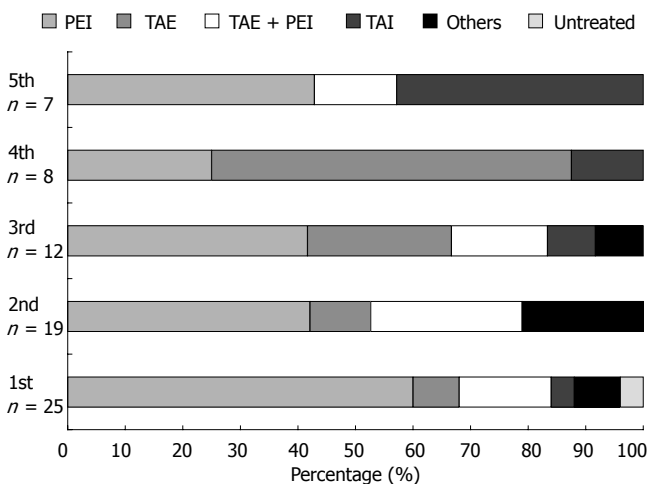


Figure 3 Treatment for repeated recurrences in patients with HCC initially treated by PEI.

(60.0%); transcatheter chemoembolization (TACE) in two (8.0%); TACE + PEI in four (16.0%); transcatheter arterial infusion chemotherapy in one (4.0%); and other methods in two (8.0%). One patient (4.0%) was untreatable because of severe liver dysfunction or poor general condition. Second, third, fourth and fifth recurrent lesions were treated with PEI in eight (42.1%), five (41.6%), two (25.0%) and three (42.8%) patients, respectively (Figure 3).

DISCUSSION

In a multicenter trial conducted in Italy^[7], the 3-, 5- and 7-years survival rates after PEI in patients with a single

Table 6 Recurrence of HCC initially treated by PEI (*n* = 25)

Location of recurrence	
Same segment	8 (32.0%)
Same and other segments	7 (28.0%)
Other segments	8 (32.0%)
Others ¹	2 (8.0%)
Tumor diameter at detection (mm)	
≤ 10	8 (32.0%)
11-15	9 (36.0%)
16-20	4 (16.0%)
21-30	2 (8.0%)
≥ 31	0
Others	2 (8.0%)

¹In two cases, tumor thrombus was seen in portal vein and HCC metastasizing to distant site (sacrum and lumbar vertebra), respectively.

HCC ≤ 30 mm in diameter have been reported as 78%, 54% and 28%, respectively; those in patients with a single HCC 30.1-50 mm as 61%, 32% and 16%, respectively; and those in patients with multiple lesions as 51%, 21% and 0%, respectively. In a series of 270 patients in Japan with fewer than three small lesions (≤ 30 mm in diameter) of HCC, overall 3- and 5-years survival rates after PEI were 81.6% and 60.3%, respectively, but the rates were higher, 87.3% and 78.3% in Child-Pugh class A patients with a solitary tumor ≤ 20 mm in diameter^[8]. The overall 3-, 5-, 7- and 10-years survival rates after PEI in the present study were 74.1%, 49.9%, 27.2% and 14.5%, respectively, in our population with minimum-sized HCC. Patients of Child-Pugh class A had 5-, 7- and 10-years survival rates of 76.0%, 42.2% and 15.8%, respectively. The outcome in this study was superior, or at least equal to that reported in other studies.

The superiority or equality of our results, including those for multinodular HCC, compared with other studies can be explained by tumor size alone. Generally speaking, the therapeutic effect of PEI is largely dependent on tumor size^[9]. Strictly speaking, the difference between tumors 15 and 16-20 mm in diameter is very important. Pathological events identified in 106 small resected HCCs < 20 mm in diameter have demonstrated local metastases (located ≤ 10 mm from the nodule), and microscopic portal invasion among the most frequently occurring tumors (the so-called distinctly nodular types). The frequency of portal invasion has been reported as significantly higher in HCC 16-20 mm in diameter (40%) than in HCC 11-15 mm in diameter (25%, *P* < 0.01)^[9,10]. In the current study, survival was not influenced by sex, age, HBsAg positivity, anti-HCV positivity, AFP levels, arterial blood flow, portal blood flow, histological characteristics, tumor multiplicity, or tumor size. However, a statistically significant difference was observed in long-term survival probability attributed only to liver dysfunction.

PEI-treated patients of Child-Pugh class A had longer survival than those of class B, which was comparable with the Italian and Japanese studies^[7,8,11]. Generally speaking, in treating HCC, prognosis depends not only on the grade of cancer spread (tumor stage)^[12], but also on the grade of residual liver function (liver disease stage). Kudo *et al*^[13] have proposed a prognostic staging system for HCC called

the Japan Integrated Staging Score (JIS score), and have suggested that the prognosis of stage I HCC (solitary, < 20 mm in diameter, no vascular invasion) depends on liver function. Here, the long-term results of PEI were equivalent to those achieved with patients treated by hepatic resection. According to the Liver Cancer Study Group of Japan^[14], the 5- and 7-years survival rates among 3674 patients with single, clinical stage I HCC lesions < 20 mm in diameter, treated by hepatic resection, reached 65.4% and 47.1%, respectively. In our study, the respective rates of 77.3% and 43.0% were obtained by restricting the final analysis to a selected group of 20 patients with single or multiple HCC nodules \leq 15 mm in diameter and with Child-Pugh class A cirrhosis. Hence, although no prospective randomized trials comparing PEI versus surgery have been conducted, the long-term results of the two treatments seem to be quite similar.

Recently, new thermal therapeutic techniques for HCC have been developed, including RFA, laser microwaves and cryotherapy. Among these, RFA has attracted much international interest and is now widely used in clinical practice. In a comparison of PEI and RFA in 86 patients with 112 HCCs^[15], a complete response was reached in 90.3% by RFA and 80% by PEI, with an average of 1.2 sessions for RFA and 4.8 sessions for PEI. However, more complications arose by RFA: one severe (hemothorax that required drainage) and four minor (intraperitoneal bleeding, hemobilia, pleural effusion and cholecystitis), compared with none by PEI.

Recently, three randomized studies which compared RFA versus PEI for first-line treatment of early-stage HCC have been published^[16-18]. European groups have failed to show a statistically significant difference in overall survival between patients who received RFA and PEI^[16]. On the other hand, survival advantages have been identified in studies in Japan and Taiwan^[17,18]. In Japan, Shiina *et al.*^[17] have described 232 patients, 118 treated by RFA and 112 by PEI. Four-year survival rate was 74% (95% CI: 65-84) for RFA and 57% (95% CI: 45-71) for PEI. RFA had a 46% smaller risk of death [adjusted relative risk, 0.54 (95% CI: 0.33-0.89), $P = 0.02$], a 43% smaller risk of overall recurrence [adjusted relative risk 0.57 (95% CI: 0.41-0.80), $P = 0.0009$], and an 88% smaller risk of local tumor progression [relative risk, 0.12 (95% CI: 0.03-0.55), $P = 0.006$] than PEI. Similarly, benefits in survival were also suggested in a subgroup analysis of a trial in Taiwan^[18].

Most trials comparing RFA and PEI for treatment of small HCC have yielded better survival, local efficacy, local recurrence and duration of treatment in favor of RFA; the only advantage in favor of PEI being a slightly lower rate of complications^[16]. In the present study, the frequency of HCC recurrence was considered to be high, with new lesions appearing in 25 of the 31 patients. However, after PEI, almost all recurrence was caused by the emergence of new nodular lesions in hepatic segments other than at the locations of the treated tumors, and were therefore probably unrelated to the original tumor. Repeat PEI alone was feasible for recurrence in 60.0%, 42.1%, 41.6%, 25.0% and 42.8% of these cases at detection of the first to fifth recurrence, respectively. As the number of lesions also

increases whenever a tumor recurs, local treatment such as PEI is of limited value, and PEI must be replaced by another form of treatment such as TACE or transcatheter infusion chemotherapy.

All the studies mentioned above confirm the high efficacy of PEI in the treatment of minimum-sized HCC. In conclusion, to achieve the best possible prognosis in its treatment, early detection of HCC < 15 mm in diameter by imaging and histological diagnoses, and early treatment by PEI are essential.

COMMENTS

Background

In treatment of hepatocellular carcinoma (HCC), only 20%-30% of patients are candidates for surgery. Thus, various non-surgical therapies, such as percutaneous ethanol injection (PEI), microwave coagulation and radiofrequency ablation (RFA) have been widely used for small HCC. Although PEI was used as first-line treatment in some Japanese and Italian centers in the 1980s and 1990s when surgical techniques were precluded, RFA has recently emerged as a real competitor to PEI. At this time, there are no unequivocal data to back up PEI as a replacement for resection as a first-line treatment for patients with early-stage HCC.

Research frontiers

PEI is a standard therapy. However, there has been a drastic shift from PEI to RFA since the introduction of the latter into clinical practice, because efficacy seems more reproducible in RFA than in PEI and microwave coagulation. Most trials comparing RFA and PEI for the treatment of small HCC have yielded not only local efficacy but also survival in favor of RFA. In addition, RFA requires shorter hospitalization than PEI, which improves quality of life. However, severe complications arise with RFA, such as hemothorax, intraperitoneal bleeding, liver abscess, liver infarction and diaphragmatic hernia, compared with none with PEI.

Innovations and breakthroughs

PEI can be safely performed. In fact, severe complications such as intraperitoneal bleeding, liver abscess, liver infarction and diaphragmatic hernia did not occur in our study. After PEI however, almost all recurrence was caused by the emergence of new nodular lesions in hepatic segments other than at the locations of the treated tumors. In those cases, PEI was used not only for the initial treatment of small HCC, but also for recurrent lesions at untreated sites after treatment. The post-PEI survival rates in our patients with Child-Pugh class A cirrhosis were at least equal to those in the post-surgery group. Tumor size (< 15 mm in diameter) and liver function (Child-Pugh class A cirrhosis) were significant survival predictors, and such patients were the best candidates for percutaneous ablation. PEI is considered not to compete with but to be complementary to RFA in the treatment of small HCC, because of its excellent safety and efficacy.

Applications

RFA is superior to PEI in the treatment of small HCC from the viewpoint of treatment response and long-term survival. PEI however, seems feasible, efficacious and is very safe. RFA is difficult with tumors located near the gall bladder, bile ducts and diaphragm. Therefore, the usefulness and importance of PEI for HCC, especially for small-sized (< 15 mm in diameter) HCC, should be emphasized. Early detection of HCC < 15 mm by imaging and histological diagnosis and early treatment by PEI are essential.

Terminology

Small HCC: < 15 mm in diameter. Local ablation therapy: non-surgical imaging-guided therapy (using US and/or CT) such as PEI, microwave coagulation and RFA. PEI: absolute ethanol is injected directly into lesions through 21-22-G needles, which are inserted under US guidance. It can destroy a considerably large volume of tissue in one session. RFA: electrodes are inserted into the tumor under imaging guidance. Radiofrequency energy is emitted from the exposed portion of the electrode, which is converted into heat and causes necrosis of the tumor. Child-Pugh class A liver cirrhosis: Cirrhosis with relatively good liver function (bilirubin < 2 mg/dL, albumin > 3.5 g/dL, and prothrombin time > 80%), without ascites and encephalopathy.

Peer review

This study reported a small cohort of 31 patients with HCC < 15 mm in diameter treated by PEI. Overall survival of the patients was equal or possibly slightly superior to that with treatment by hepatic resection or radioablation. Although a small number of patients was analyzed, it represents an interesting and potentially important clinical finding.

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Anti-cancer and anti-angiogenic effects of curcumin and tetrahydrocurcumin on implanted hepatocellular carcinoma in nude mice

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Abstract

AIM: To determine the effect of tetrahydrocurcumin (THC) on tumor angiogenesis compared with curcumin (CUR) by using both *in vitro* and *in vivo* models of human hepatocellular carcinoma cell line (HepG2).

METHODS: The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay was used for testing the anti-proliferating activities of CUR and THC. In male BALB/c nude mice, 2×10^6 human HepG2 cells were inoculated onto a dorsal skin-fold chamber. One day after HepG2 inoculation, the experimental groups were fed oral daily with CUR or THC (300 mg/kg or 3000 mg/kg). On d 7, 14 and 21, the tumor microvasculature was observed using fluorescence videomicroscopy and capillary vascularity (CV) was measured.

RESULTS: Pathological angiogenic features including microvascular dilatation, tortuosity, and hyper-permeability were observed. CUR and THC could attenuate these pathologic features. In HepG2-groups, the CV were significantly increased on d 7 (52.43%), 14 (69.17%), and 21 (74.08%), as compared to controls (33.04%,

$P < 0.001$). Treatment with CUR and THC resulted in significant decrease in the CV ($P < 0.005$ and $P < 0.001$, respectively). In particular, the anti-angiogenic effects of CUR and THC were dose-dependent manner. However, the beneficial effect of THC treatment than CUR was observed, in particular, from the 21 d CV (44.96% and 52.86%, $P < 0.05$).

CONCLUSION: THC expressed its anti-angiogenesis without any cytotoxic activities to HepG2 cells even at the highest doses. It is suggested that anti-angiogenic properties of CUR and THC represent a common potential mechanism for their anti-cancer actions.

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Key words: Tumor angiogenesis; HepG2; Curcumin; Tetrahydrocurcumin; Intravital fluorescence videomicroscopy

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INTRODUCTION

Hepatocellular carcinoma (HCC) is a highly malignant tumor characterized by active neovascularization^[1]. Since HCC recruit new blood vessels to support tumor growth, an anti-angiogenic agent is one of the goal drugs to treatment of HCC. Cancer cells have a very high rate of mutation in contrast to endothelial cells, which are the main components of blood vessels and have a lower rate of mutation. Because of this genetic stability, anti-cancer treatment inducing tumor-induced angiogenesis is expected to be less vulnerable to such drug tolerance. Moreover, it may work on a broad spectrum of solid tumors because all these tumors need to induce angiogenesis for their processes. It is of great interest to apply the idea of anti-angiogenesis treatment to the prevention of cancer. If

food factors that can inhibit angiogenesis were to be found, such factors could be used to stop small cancers from progression. Curcumin (CUR) is considered to be among such candidates. Curcumin (diferuloylmethane) is a phenolic compound from the plant *Curcuma longa*. A variety of pharmacological effects of curcumin have been reported, including anti-inflammatory^[2], anti-oxidant^[3,4], and anti-carcinogenic activities^[5-7]. Recently, it has been shown that the anti-cancer property of curcumin is mediated in part by its anti-angiogenic activity^[8-11]. As the active metabolite of curcumin obtained in gastrointestinal tract, tetrahydrocurcumin (THC) is a reduced analog of curcumin with phenolic and β -diketo moieties as well as curcumin (Figure 1). Sugiyama *et al*^[12] demonstrated that THC exhibited similar physiological and pharmacological properties, in particular, THC has possessed strong anti-oxidant action than curcuminoids including curcumin, demethoxycurcumin, and bisdemethoxycurcumin^[13]. Although the role of THC in anti-cancer activity has been implicated^[13], its possible mechanism(s) and efficacy related to curcumin anti-cancer responsibility are still controversial. For instance, THC has been reported to be a less effective chemopreventive agent in mouse skin than curcumin^[14]. In contrast, 0.5% THC mixed diet showed a stronger inhibitory effect on 1,2-dimethylhydrazine induced mouse colon carcinogenesis than curcumin^[15]. Therefore, the present study was aimed to determine the effect of THC on tumor angiogenesis in comparison with curcumin by using both *in vitro* and *in vivo* models of human hepatocellular carcinoma cell line (HepG2).

MATERIALS AND METHODS

Preparation of curcumin and THC

The curcuminoid mixture obtained from the rhizomes of *Curcuma longa* was subjected to silica gel column chromatography, using hexane-dichloromethane, dichloromethane and dichloromethane-methanol as eluents to afford curcumin (CUR) as the major constituent. Recrystallization was accomplished by dissolving the evaporated eluate with a small quantity of dichloromethane and ethanol was then added. CUR crystallized out as yellow needles, melting point (m.p.) 181-183°C. THC was synthesized from CUR by catalytic hydrogenation reaction, with palladium on charcoal as a catalyst. The product was purified by silica gel column chromatography followed by recrystallization with dichloromethane-hexane to give 75% yield of THC as colorless needles, m.p. 93-94°C. The spectroscopic (IR, ¹H-NMR and mass spectra) data of the synthesized THC were consistent with the reported values^[16].

In vitro study of anti-proliferation assay

The effects of CUR and THC on the growth and survival of human hepatocellular carcinoma cell lines were measured using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay. Briefly, HepG2 cells (7.5×10^4 per well) were plated in 0.2 mL medium containing 10% FBS in triplicate in 96 well plate after 24 h medium was removed and then treated with 0.2 mL medium containing the indicated concentrations of CUR or THC at 37°C

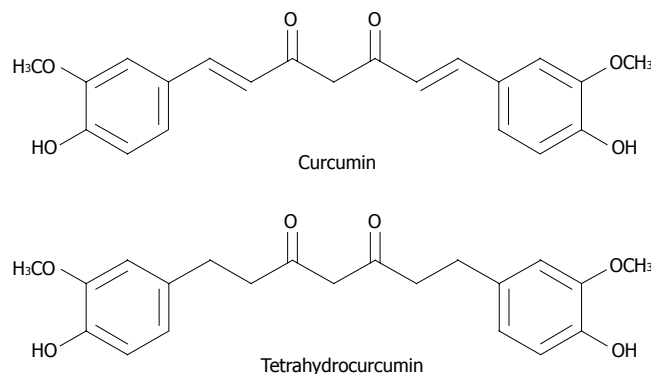


Figure 1 Chemical structures of CUR and THC. CUR and THC have similar β -diketo and phenolic moieties.

for 24 h. At the end of incubation, 0.050 mL of MTT solution (5 mg/mL) was added to each well. After 20 min incubated at 37°C, 0.030 mL of isopropanol was added to dissolve the formazan crystals. The absorbance of the MTT formazan was determined at 570 nm in an enzyme-linked immunosorbent assay (ELISA) reader. Cell growth index was defined as a percentage of the absorbance of treated cells to untreated cells.

Animal preparation

The experiments were performed in BALB/c-nude mice (b.w. 20-25 g; $n = 90$). The animal experiment was conducted according to the guideline of experimental animals by The National Research Council of Thailand (1999). The mice were bred and maintained in a specific pathogen germ-free environment.

The mice were divided into four groups: (1) normal (control) mice with vehicle treatment (Con, $n = 15$), (2) HepG2-induced tumor mice (HepG2, $n = 15$), (3) HepG2-induced tumor mice with CUR treatment (HepG2-CUR, $n = 30$) and (4) HepG2-induced tumor mice with THC treatment (HepG2-THC, $n = 30$). In order to implant HepG2 a dorsal skin-fold chamber (7 mm diameter)^[16] was used. After the anesthetization by sodium pentobarbital (50 mg/100 g BW, i.p.), 30 μ L of 2×10^6 HepG2 cells were inoculated in the middle area of dorsal skin-fold chamber and then covered with 7 mm glass slip. All surgical procedures were performed under aseptic conditions. The animals were then housed one animal per cage with free access to sterile water and standard laboratory chow.

In the CUR and THC treated groups (HepG2-CUR and HepG2-THC groups), the mice were daily oral treated by 2 mL of 300 and 3000 mg/kg BW CUR and THC dissolved in 0.1% dimethyl sulfoxide (DMSO; Sigma, USA). These treatments were started twenty-four hours after the inoculation. In the control group (Con and HepG2), the mice received vehicle (0.1% DMSO) instead.

Intravital fluorescence videomicroscopy study

The experiments were performed d 7, 14 and 21 after vehicle, CUR or THC treatments. The mice were anesthetized with an intraperitoneal injection of sodium pentobarbital (50 mg/kg BW). A catheter was inserted into



Figure 2 An example of 5 windows selected for measurement of capillary vascularity (CV) from the video-image at a low magnification ($\times 10$, Bar = 100 μm).

a jugular vein for application of fluorescence tracers. Then, the dorsal skin-fold chamber was removed and skin area around the chamber was fixed with modeling wax on a plate.

The microcirculation within a studied area was observed under an intravital fluorescence microscope using a $10\times$ objective. During the experiment, a videocamera (Sony, Japan) was used to project the image onto a monitor (Sony, Japan) and to record the interested areas within the tumor-bearing chambers by using a video-recorder (Sony, Japan). The videotape of each experiment was then analyzed off-line using digital image processing software (Global Lab II).

For visualization of the microvascular lumen, a bolus of 0.1 mL of 5% fluorescein isothiocyanate-labeled dextran (FITC-dextran) was injected into the jugular vein 5 min prior to the recording. The recorded videoimages were analyzed and calculated for capillary vascularity, using digital image processing software (Global Lab II) and then expressed in percentage as described previously^[9]. The capillary vascularity (CV) level was used as an index of angiogenesis.

Measurement of capillary vascularity

Based on the recorded video image, we measured capillary vascularity (CV) defined as follows: $\text{CV} = (\text{number of pixels within the capillaries}) \times 100 (\%) / \text{total number of pixels within the selected window area}$.

In each mouse, we observed and recorded at 5 positions on the surface of tumors by moving the microscopic stage. Figure 2 shows an example of 5 windows selected on one low-magnified image. Each window (video frame of 100×100 pixels) was selected so as to cover any no large vessel. By determining both minimum and maximum intensities of pixels, we counted the total number of pixels over all capillaries in each window, using digital image processing software (Global Lab II) and expressing the CV as percents of capillary area to total area. Averaging the CV's ($\text{CV}_i, i = 1-5$) measured over 5 video-frames (positions), we calculated the mean CV in one mouse: $\text{Mean CV} = (1/5) \sum \text{CV}_{(1-5)}$.

Statistical analysis

Results were shown as mean \pm SE. One-way ANOVA was used to evaluate the difference of means. The statistical differences were considered at the probability level (P value) of less than 0.05.

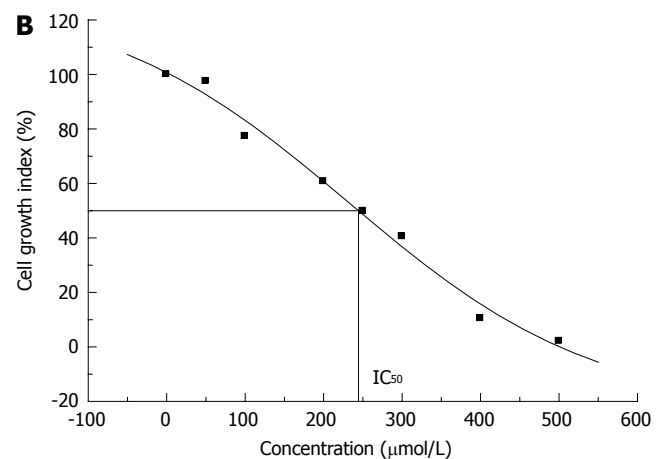
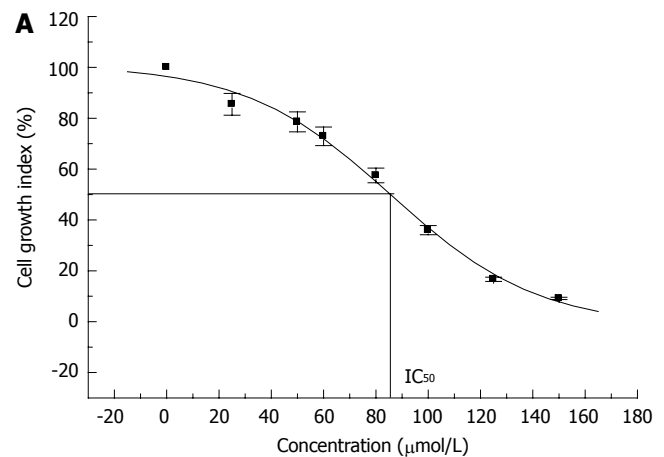


Figure 3 IC_{50} and cell growth index by MTT assay. **A:** Effects of CUR on cell proliferation of HepG2 cell; **B:** Effects of THC on cell proliferation of HepG2 cell. Values given represent the mean \pm SE of three independent experiments carried out in triplicate. The IC_{50} value was required to decrease viability from 100% to 50%.

RESULTS

Anti-proliferation effects of curcumin and its analog on HepG2 cells

The anti-proliferation activity of CUR and its analog, THC, were examined in HepG2 cell lines by MTT assay. It was found that CUR is a more potent anti-proliferative agent than THC. The IC_{50} of CUR and THC were 85.98 and 233.12 $\mu\text{mol/L}$, respectively (Figure 3).

Tumor angiogenesis in HepG2-implemented nude mice

Intravital fluorescence microscopic observation demonstrated a number of neocapillaries in HepG2 groups. Figure 4 shows fluorescence videoimages of the microvasculature for control and HepG2 groups on d 7, 14 and 21 after tumor cells implantation. In addition, pathological angiogenic features including abrupt changes in the diameter, tortuosity, and hyper-permeability were also observed in HepG2 groups.

Effects of curcumin and its analog on tumor angiogenesis in HepG2-implemented nude mice

Figure 5 demonstrated the intravital fluorescent microscopic observation of tumor angiogenesis affected by CUR and THC treatment. The result showed that the appearance of

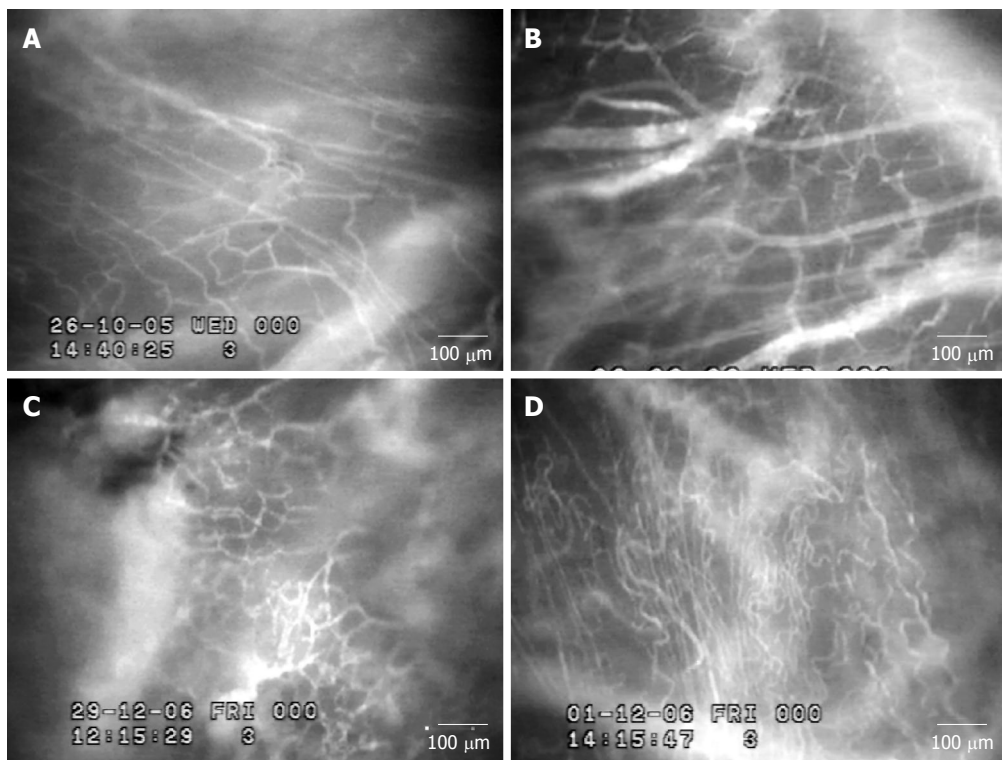


Figure 4 A: Fluorescence videomicroscopy image of the microvasculature for control; B: Fluorescence videomicroscopy image of the microvasculature for 7 d HepG2 groups; C: Fluorescence videomicroscopy image of the microvasculature for 14 d HepG2 groups; D: Fluorescence videomicroscopy image of the microvasculature for 21 d HepG2 groups ($\times 10$, Bar = 100 μm).

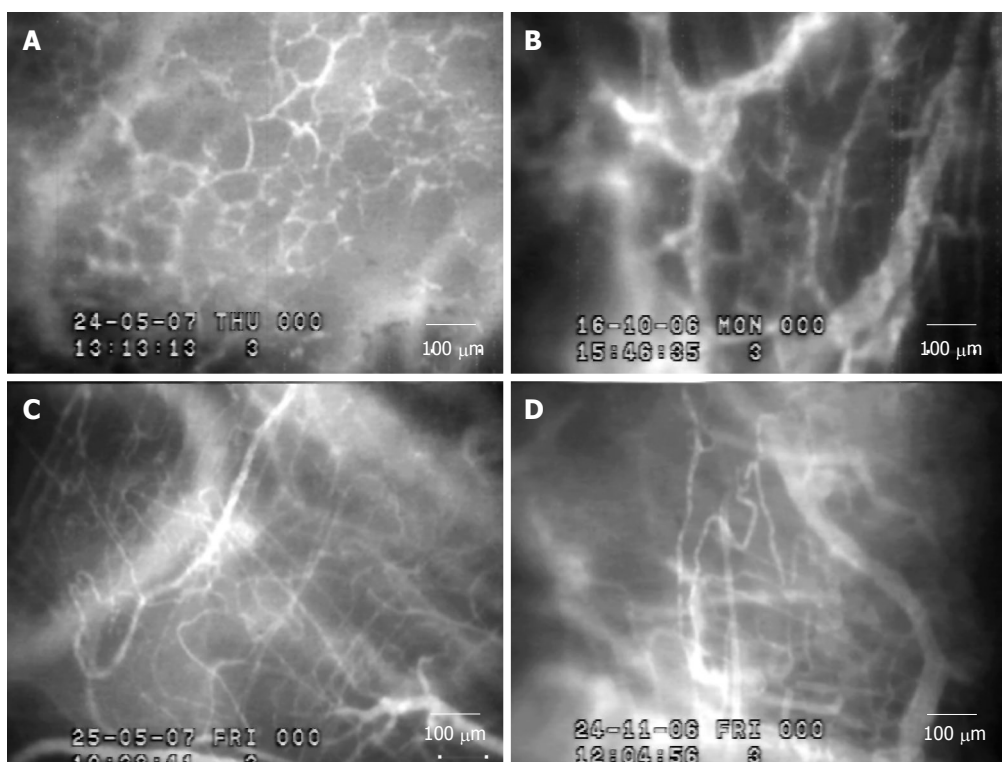


Figure 5 A: Fluorescence videomicroscopy image of capillary vascularity on 14 d after the implantation of tumor cells with treatment of CUR (3000 mg/kg); B: Fluorescence videomicroscopy image of capillary vascularity on 14 d after the implantation of tumor cells with treatment of THC (3000 mg/kg); C: Fluorescence videomicroscopy image of capillary vascularity on 21 d after the implantation of tumor cells with treatment of CUR (3000 mg/kg); D: Fluorescence videomicroscopy image of capillary vascularity on 21 d after the implantation of tumor cells with treatment of THC (3000 mg/kg, $\times 10$, Bar = 100 μm).

neocapillaries induced by HepG2 was markedly reduced on 14 and 21 d after treatment of CUR and THC (3000 mg/kg BW). In addition, the abnormalities of neocapillary network pattern were attenuated after both treatments.

Capillary vascularity of tumor tissue in HepG2-implanted nude mice

For analysis of microvascular parameters, CV in the surface

area of tumor was calculated at different periods after tumor cell inoculation. Figure 6 shows CV of 7, 14 and 21 d after vehicle, CUR or THC treatment in control and HepG2 groups. In HepG2-group, the percentage of CV was significantly increased on d 7 (52.43%), 14 (69.17%), and 21 (74.08%), as compared to age-matched controls (33.04%, $P < 0.001$). Treatment with CUR and THC showed significant decrease in the percentage of CV ($P < 0.005$

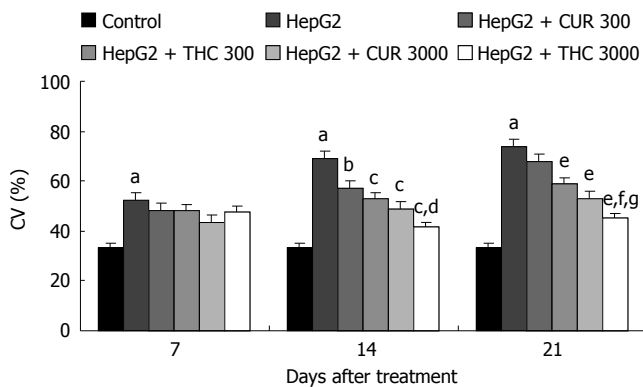


Figure 6 Capillary vascularity (mean \pm SE) of 7 d, 14 d, and 21 d after vehicle (0.1% DMSO), CUR or THC (300 and 3000 mg/kg BW) treatment in control and HepG2 groups. ^a $P < 0.001$, vs control group with vehicle; ^b $P < 0.005$, vs 14 d HepG2 group with vehicle; ^c $P < 0.001$, vs 14 d HepG2 group with vehicle; ^d $P < 0.01$, vs 14 d HepG2-THC 300 group; ^e $P < 0.001$, vs 21 d HepG2 group with vehicle; ^f $P < 0.001$, vs 21 d HepG2-THC 300 group; ^g $P < 0.05$, vs 21 d HepG2-CUR 3000 group.

and $P < 0.001$, respectively). In particular, the anti-angiogenic effects of CUR and THC were dose-dependent manner. However, the beneficial effect of (3000 mg/kg) THC treatment than CUR was suggested, in particular, from the 21 d percent of neocapillaries density (44.96% and 52.86%, $P < 0.05$).

DISCUSSION

By using MTT assay, the anti-proliferation properties of CUR and THC were examined in HepG2 cell lines. It was found that CUR has more potent anti-proliferation properties than THC. The IC_{50} of CUR and THC were 85.98 and 233.12 μ mol/L, respectively. CUR has been shown to inhibit cell proliferation in a wide variety of human cancer cell lines *in vitro*^[18] and in various xenotransplant and orthotopic models of human cancer in rodent^[18,19]. CUR suppresses the activation of several transcription factors that are implicated in carcinogenesis^[18], including nuclear factor kappa B (NF- κ B)^[20], activator protein 1 (AP-1)^[21], and at least two of the signal transducer and activator of transcription proteins (STAT3, STAT5), and modulates the expression of early growth response protein 1 (Erg-1), peroxisome proliferators-associated receptor gamma (PPAR- γ)^[22]. It also suppresses the expression of cyclin D1^[23] and induces apoptosis of tumor cells^[24,25]. According to the inhibitory effects of CUR on these cell signaling pathways, CUR may mediate its anti-proliferation by inhibiting either expression or activation of proteins that required for cell survival or cell proliferation. Furthermore, it might imply that THC could be able to suppress these key factors of signaling pathways at lesser efficacies than CUR.

By using intravital fluorescence videomicroscopy, the results showed that: (1) there was a significant increase in the numbers of CV with the heterogeneous network in HepG2 groups as compared to controls as which consistency with our previous reports^[9,10]. (2) In this study, it was confirmed that more neocapillary density was observed as a time-dependent manner during tumor

progression (CV of 21 d > CV of 14 d > CV of 7 d). (3) THC is a stronger anti-angiogenic agent than CUR.

Therefore, in the present study, CUR and THC could exert both *direct* and *indirect* actions by inhibiting tumor cell proliferation and by inhibiting tumor angiogenesis, respectively. Although the precise mechanisms that lead to tumor angiogenesis are not fully understood, several studies have shown that tumor angiogenesis which is the common process necessary for every tumor types requires the expressions of cyclooxygenase-2 (COX-2), vascular endothelial growth factor (VEGF), and matrix metalloproteinase-9 (MMP-9). Similar to our findings, CUR has been shown to suppress the proliferation of human vascular endothelial cells *in vitro*^[26] and to abrogate angiogenic response *in vivo*^[10]. It has also been shown that CUR inhibited Akt activation and down-regulated the expression of 5-lipoxygenase^[20].

The significant finding initiated from the current study is that THC has efficacy in anti-angiogenic activity than CUR. Although THC is shown to be less anti-proliferative activity than CUR, several studies agreed to demonstrate that THC is a more potent anti-oxidant than CUR^[12,27], and the mechanism could be implied by its β -diketo moiety^[12]. A number of evidence also suggested that tumor-mediated inflammatory response could generate an intensive local accumulation of reactive oxygen species (ROS). ROS may play a role as the mediator for the consequence of tumor induced the expression of tumor biomarkers involved tumor angiogenesis. The activation for VEGF and angiopoietin-1 induced EC migration and/or proliferation through an increase in ROS mainly proposed by a number of researchers^[28,29]. It was found that ethanol stimulated actin cytoskeletal reorganization, cell motility and tube formation in a ROS-dependent manner in ECs^[30]. Furthermore, Leptin, a circulating adipocytokine, upregulated VEGF mRNA and stimulates cell proliferation through an increase in ROS in ECs^[31].

Based on the idea of ROS, several other antioxidants such as green tea catechins, vitamin E, and natural polyphenols from red wine have been documented as the inhibitors of tumor angiogenic responses^[32]. According to the more potent anti-oxidant activity of THC, the more potent anti-angiogenic activity of THC than CUR could be used to describe the different anti-angiogenic results.

Finally, it was concluded that both CUR and THC have shown to produce both anti-proliferation and anti-angiogenesis at different manner. More potent tumor anti-angiogenesis was observed for THC, and it might be due to its higher anti-oxidant activity than CUR. It is implied that THC might be a promising candidate for tumor anti-angiogenesis in the near future.

COMMENTS

Background

Anti-angiogenesis, the postulated mechanism of anti-cancer activities of tetrahydrocurcumin (THC) and curcumin (CUR), was examined using hepatocellular carcinoma cells (HepG2)-implanted nude mice. CUR has been found to be an angiogenic inhibitor. However, THC, a potent anti-oxidative agent is responsible for the reported effect is still to be determined. The present study was aimed to determine the effect of THC on tumor angiogenesis in comparison with CUR by using both *in vitro* and *in vivo* models of HepG2.

Research frontiers

THC contains both a phenolic moiety and a β -diketone moiety in the same structure. THC exhibits many of the same physiologic and pharmacological activities as CUR and in some systems may exert greater anti-oxidant activity than CUR. Therefore, the role of THC in preventive and therapeutic of cancer is gain interest. Anti-angiogenic therapy is one of the most promising strategies for cancer treatment. In this study, anti-angiogenic activity was investigated by evaluating the density of neovascularization induced by Hepatocellular carcinoma cell (HepG2) in nude mice, using intravital fluorescence videomicroscope. The results showed that THC exerts significant anti-angiogenic activity. Therefore, THC might be a promising candidate for tumor anti-angiogenesis in the near future.

Innovations and breakthroughs

The present study showed the anti-cancer and anti-angiogenic activities of CUR and THC. CUR and THC have shown to produce both anti-proliferation and anti-angiogenesis at different manner. More potent tumor anti-angiogenesis was observed for THC, and it might be due to its higher anti-oxidant activity than CUR.

Applications

The findings from this study support the idea that anti-oxidative substances can be a therapeutic target for treating cancer.

Peer review

In this study, THC, a novel type of anti-oxidant showed anti-angiogenic activities without any cytotoxic effect. Importantly, our results have provided originally an *in vivo* evidence for anti-angiogenic activity of THC, in particular by using hepatocellular-carcinoma inoculated skin-chamber model.

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VIRAL HEPATITIS

Serial changes in expression of functionally clustered genes in progression of liver fibrosis in hepatitis C patients

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Abstract

AIM: To investigate the relationship of changes in expression of marker genes in functional categories or molecular networks comprising one functional category or multiple categories in progression of hepatic fibrosis in hepatitis C (HCV) patients.

METHODS: Marker genes were initially identified using DNA microarray data from a rat liver fibrosis model. The expression level of each fibrosis associated marker gene was analyzed using reverse transcription-polymerase chain reaction (RT-PCR) in clinical biopsy specimens from HCV-positive patients ($n = 61$). Analysis of changes in expression patterns and interactions of marker genes in functional categories was used to assess the biological mechanism of fibrosis.

RESULTS: The profile data showed several biological changes associated with progression of hepatic fibrosis. Clustered genes in functional categories showed sequential changes in expression. Several sets of clustered genes, including those related to the extracellular matrix (ECM), inflammation, lipid metabolism, steroid metabolism, and some transcription factors important for hepatic biology showed expression changes in the immediate early phase (F1/F2) of fibrosis. Genes associated with aromatic amino acid (AA) metabolism, sulfur-containing AA metabolism and insulin/Wnt signaling showed expression changes in the middle phase (F2/F3), and some genes related to glucose

metabolism showed altered expression in the late phase of fibrosis (F3/F4). Therefore, molecular networks showing serial changes in gene expression are present in liver fibrosis progression in hepatitis C patients.

CONCLUSION: Analysis of gene expression profiles from a perspective of functional categories or molecular networks provides an understanding of disease and suggests new diagnostic methods. Selected marker genes have potential utility for biological identification of advanced fibrosis.

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Key words: Hepatitis C; Liver fibrosis; Marker gene; Gene expression; RT-PCR; Molecular network; Metabolism; Transcription factor; Diagnosis

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INTRODUCTION

Liver fibrosis is caused by liver disorders such as hepatitis C, hepatitis B, alcoholic hepatitis and non-alcoholic hepatitis. Fibrosis progresses gradually and finally disrupts liver structure and function over several decades, leading to fatal diseases such as cirrhosis and hepatocellular carcinoma (HCC). Classification of fibrosis progression is usually based on histological criteria using the METAVIR scoring system^[1], which includes five stages: F0 (no fibrosis), F1, F2, F3, and F4 (cirrhosis). Such a classification is essential in decisions regarding treatment of liver fibrosis. Prominent subjective symptoms do not occur from F1 to F3, but patients begin to be aware of symptoms after F4. However, the F4 stage of cirrhosis is almost incurable and diagnosis of fibrosis at an earlier stage is desirable. The biology after F4 (cirrhosis) has been well studied, due to the interest in diagnosis and therapy for hepatocellular carcinoma (HCC),

but progression to HCC may begin in the early stage of fibrosis in hepatitis C^[2].

Prevention of HCC and inhibition of fibrosis in the early phase is important, but the detailed hepatic biological changes corresponding to the F stage are unclear. To understand the background of the early stage of fibrosis, we previously identified genes that can be used as markers of biological changes in progression of hepatic fibrosis and diagnosis of fibrotic progression; these data were obtained from DNA microarray data from an experimental DMN (dimethylnitrosamine)-treated rat model of hepatic fibrosis^[3]. This work led to marker genes that were arranged in functional categories related to fibrosis, based on genes associated with hepatic cell types such as Kupffer cells, hepatic stellate cells, and hepatocytes.

These marker genes give information on cell-specific and time-dependent behavior of each hepatic cell in fibrogenesis. In the current work, the behavior of marker genes associated with a particular F stage was analyzed using RT-PCR in clinical biopsy specimens from hepatitis C patients. This profile data revealed several biological changes in progression of hepatic fibrosis. Since many functionally clustered genes showed similar changes in expression, we propose serial expression changes in molecular networks associated with liver fibrosis progression in hepatitis C patients. Many functionally clustered genes showed large changes in expression in the early stage of fibrosis, suggesting the importance of therapy at this early stage. Alteration of gene expression also suggested qualitative changes in biological status in the transition from F3 to F4, which is a suspected risk factor for development of HCC. We conclude that analysis of gene expression profiles from a perspective of molecular networks provides improved understanding of disease and indicates potential methods of diagnosis.

MATERIALS AND METHODS

Patients in the clinical study

All patients were recruited from the Osaka City University Hospital (Osaka, Japan). Sixty-one patients with seropositive results in diagnosis using the third-generation hepatitis C virus enzyme-linked immunosorbent assay (Lumipulse II Ortho HCV, Ortho-Clinical Diagnostics, Tokyo, Japan) and positive serum HCV-RNA were included in the study. Informed consent was obtained from all patients. Liver biopsies were performed on all patients enrolled in the study, and the histological features of the liver specimens were analyzed and graded using the METAVIR scoring system^[1]. The liver fibrotic stage (F stage) and inflammatory activity (A grade) were determined histologically: at least four subjects were found to be in each F stage classification. Determination for chymase 1 exceptionally has been done with three subjects in F4 stage due to the lack of appropriate samples.

Part of the biopsy sample from each patient was immediately immersed in RNAlater (QIAGEN, The Netherlands) to inhibit RNAase and then kept at 4°C overnight before being transferred to another tube and frozen at -80°C.

RT-PCR analysis

Total RNA was extracted from liver biopsy samples using

an ISOGEN kit (Nippongene) and reverse transcribed using a High Capacity cDNA Archive Kit (ABI, Foster City, CA), in each case according to the manufacturer's instructions. The total RNA in the final reaction mixture was 10 ng/μL. Real-time PCR was performed on an Applied Biosystems 7500 Real-Time PCR System (ABI) data collection system, and analyses were performed using the accompanying software. RT-PCR was performed using 0.8 μL cDNA in each well, with a final concentration of 1X the probes of the TaqMan® Gene Expression Assay and 1X the Taqman Universal PCR Master Mix (ABI). The final reaction volume was 20 μL. Each sample was analyzed in duplicate. The thermal cycler conditions were 2 min at 50°C and 10 min at 95°C, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C.

Data were analyzed using the comparative CT method, in which the expression level of a target gene is normalized relative to an endogenous reference. GAPDH was used as the endogenous reference in all experiments. The target C_T and endogenous control C_T were calculated for each sample, and the target gene expression level was then calculated using the formula $2^{-(C_T - C_{CT})}$. The average of duplicate measurements was obtained, and the relative expression of each gene in a sample was calculated by setting the expression of GAPDH equal to 1000. PCR fluorogenic probes for all the target genes and the endogenous reference were purchased as TaqMan® Gene Expression Assays (ABI).

Statistical analysis

A Kruskal-Wallis test was applied to select marker genes with statistically significant changes ($P < 0.05$) in expression level during the fibrosis progression. This calculation was performed using SPSS (SPSS Inc., IL, USA). The gene expression data were subjected to hierarchical clustering analysis using Genowiz™ software (Ocimum Biosolutions).

Pathway analysis of PCR data

The behavior and relationships of marker genes in pathways associated with lipid metabolism were analyzed with bioSpace Explorer, a system for analysis of expression profile data. This system was produced collaboratively by Pharmafrontier Co. Ltd. and World Fusion Co. Ltd. to examine molecular interactions in expression profile data, using both manual and computational text mining.

RESULTS

Expression behavior of marker genes

All marker genes determined in this study are listed in Table 1. For each gene, since the quantitative limitation of biopsy specimens resulted in a difference in sample number for each probe, the type of samples, indicating the number of biopsy specimens, is listed in Table 2. Marker genes were selected as representative members of functional categories or molecular networks based on their expression changes in an experimental hepatic fibrosis model^[3]. Marker genes with statistically significant changes in expression are listed in Table 1. Genes that showed statistically insignificant changes in expression are listed with the gene name only in Table 1. Marker genes that showed statistically significant changes by *t* test during a

Table 1 Expression profiles of marker genes with statistically significant changes during fibrosis progression

Functional category	Description	Gene name	Expression at F1	Group	Expression ratio			Serial	Type
					F2/F1	F3/F2	F4/F3		
ECM (or other HSC marker)	Decorin	DCN	217.6	2	1.2	1.1	1.1	1	1
	Matrix metalloproteinase 2	MMP2	11.7	2	1.5	1.2	0.9	2	1
	Hyaluronan-mediated motility receptor	HMMR	1.2	2	2.6	0.6	1.1	3	1
	Lysyl oxidase	LOX	1.0	2	1.4	1.2	0.9	4	1
	Lysyl oxidase-like 1	LOXL1	0.8	2	1.8	1.4	1.0	5	1
	Tropomyosin 1	TPM1	35.0	2	1.8	1.5	1.1	6	1
	Prion	PRNP	19.4	3	1.2	0.7	1.0	7	1
	Collagen, type I, alpha 1	COL1A1	16.2	2	1.3	2.2	0.6	8	1
	Collagen type III alpha 1	COL3A1	143.9	2	1.3	1.5	0.8	9	1
	Collagen type alpha 1	COL4A1	16.8	2	1.3	1.9	0.6	10	1
	Humican	LUM	22.8	2	1.2	2.0	0.9	11	1
	Sialoprotein	SPP1	13.2	2	1.2	2.2	1.4	12	1
	Glypican 3	GPC3	23.5	2	1.8	2.5	1.0	13	1
	Proline 4-hydroxylase, alpha polypeptide I	P4HA1	34.0	1	1.1	0.4	1.2	14	1
Inflammation (or apoptosis)	Insignificant change: MGP, BGN, TAGLN, LGALS1, EDG2, EDG5, TNNT2								
	Lysozyme	LYZ	185.3	2	2.1	0.8	1.0	15	2
	TGF beta	TGFB1	51.9	3	1.5	0.8	0.9	16	1
	TGF beta 3	TGFB3	3.3	3	1.4	0.9	0.9	17	3
	TNF	TNF	2.6	3	1.7	0.6	1.2	18	3
	Natural killer cell proteinase 1	GZMB	1.7	3	1.6	0.5	1.1	19	1
	IL1 beta	IL1B	2.6	3	1.5	0.7	1.1	20	3
	Hemopoietic cell kinase	HCK	22.4	3	1.3	0.6	0.9	21	4
	Interleukin 6 receptor	IL6R	142.9	1	0.9	0.7	1.0	22	3
	BCL2-related ovarian killer	BOK	205.9	1	0.9	0.7	1.1	23	5
	Caspase 2	CASP2	1.3	1	1.2	0.7	0.8	24	5
	Chymase 1, mast cell	CMA1	0.2	2	0.7	1.8	2.2	25	6
	Insignificant change: LTBP1, LBP, TNFRSF1B, DEFB1, IL1RN, S100A8, BRIC3, CARD12, CASP1, CASP4, CASP8, PAWR, CD19, CD3Z, MS4A1, CD37, TRA [®]								
Growth factor	Growth hormone receptor	GHR	102.3	1	0.8	0.9	0.9	26	4
	IGF1	IGF1	56.5	1	0.9	0.7	1.1	27	1
	Insignificant change: PTN, FST, PRLR								
Insulin/ Wnt signal	Cyclin D1	CCND1	190.6	2	1.7	1.0	1.0	28	5
	Forkhead box M1	FOXM1	0.6	3	3.7	0.8	1.1	29	4
	Gap junction protein, alpha 1, 43 kDa (connexin 43)	GJA1	4.0	3	2.4	0.5	1.0	30	5
	V-akt murine thymoma viral oncogene homolog 1	AKT1	77.6	1	0.9	0.9	1.0	31	4
	V-akt murine thymoma viral oncogene homolog 2	AKT2	59.6	1	0.9	0.7	0.8	32	7
	Catenin (cadherin-associated protein), beta 1, 88 kDa	CTNNB1	159.0	1	1.1	0.7	1.0	33	5
	Catenin, beta interacting protein 1	CTNNBIP1	24.0	1	1.1	0.7	0.8	34	5
	Glycogen synthase kinase 3 beta	GSK3B	44.1	1	1.1	0.8	0.8	35	5
	Dishevelled, dsh homolog 1 (Drosophila)	DVL1	12.2	1	1.1	0.7	0.9	36	7
	Membrane-bound transcription factor peptidase, site 1	MBTPS1	72.4	1	0.9	0.7	1.0	37	7
	Membrane-bound transcription factor peptidase, site 2	MBTPS2	13.2	1	0.9	0.7	1.0	38	7
	Tribbles homolog 3 (Drosophila)	TRIB3	18.4	1	1.1	0.9	0.4	39	7
	Insignificant change: GSK3A, INSIG1, INSIG2, PRKCB1, PRKCD								
Others signal	Regucalcin (senescence marker protein-30)	RGN	697.8	1	0.9	0.8	0.8	56	4
	Insignificant change: DAB2, PMP22, S100A10, LCN2								
Transcription factors	CCAAT/enhancer binding protein (C/EBP), alpha	CEBPA	352.2	1	0.7	1.2	0.7	40	4
	Retinoid X receptor, alpha	RXRA	409.6	1	0.7	1.0	0.8	41	4
	Hepatocyte nuclear factor 4, alpha	HNF4A	708.7	1	0.7	1.1	0.9	42	4
	Transcription factor 1 (HNF1)	TCF1	26.7	1	0.8	0.9	0.8	43	4
	Nuclear receptor subfamily 0, group B, member 2	NR0B2	166.7	1	0.7	0.9	0.8	44	4
	Peroxisome proliferative activated receptor, alpha	PPARA	62.9	1	0.7	0.8	0.9	45	4
	Inhibitor of DNA binding 1 (splice variation)	ID1	463.4	3	1.6	0.5	1.4	46	1
	AE binding protein 1	AEBP1	24.6	2	1.4	1.2	1.0	47	4
	Nuclear receptor subfamily 1, group H, member 2	NR1H2 (LXRB)	9.0	1	1.2	0.7	1.0	48	7
	Nuclear receptor subfamily 1, group H, member 3	NR1H3 (LXRA)	27.8	1	1.0	0.7	0.8	49	7
	Nuclear receptor subfamily 1, group H, member 4	NR1H4 (FXR)	107.0	1	1.0	0.7	0.9	50	7
	c/EBPbeta	CEBPB	274.3	1	0.9	0.7	0.9	51	2
	Upstream transcription factor 2, c-fos interacting	USF2	323.7	1	0.9	0.7	0.9	52	4
	Estrogen-related receptor alpha	ESRRA	79.6	1	1.2	0.6	0.8	53	7
	C-met	MET	73.5	1	1.0	0.7	1.1	54	3
	Upstream transcription factor 1	USF1	30.8	1	1.0	0.9	0.8	55	4
Transporter	Insignificant change: ONECUT1, JUNB, NR3C1, PPARG, PPARGC1B, PPARGC1A, SREBF2, FHL2								
	Solute carrier family 6, member 6	SLC6A6	3.7	3	2.5	0.6	1.0	57	5
	Solute carrier family 7, member 1	SLC7A1	0.9	3	2.3	0.7	1.3	58	5
	Solute carrier family 38, member 2 Alanine-transporter)	SLC38A2	68.6	3	1.3	0.6	1.0	59	5
	Solute carrier family 25 member 15	SLC25A15	85.7	1	1.1	0.7	0.9	60	5
	Solute carrier family 7, member 7	SLC7A7	7.1	1	1.1	0.6	1.0	61	5
	Solute carrier family 17 (sodium phosphate), member 1	SLC17A1	23.6	1	0.9	0.7	1.0	62	4

Insignificant change: SLC38A3, ABCB1, SLC15A4									
Redox	Catalase	CAT	1977.7	1	0.8	0.9	1.0	63	4
	Paraoxonase 1	PON1	191.1	1	0.8	0.7	0.9	64	4
Blood	Coagulation factor X	F10	187.5	1	0.8	1.0	0.9	65	4
coagulation	Angiotensinogen	AGT	1958.4	1	0.8	1.0	1.0	66	4
	Fibrinogen, A alpha polypeptide	FGA	8337.5	1	0.6	1.0	0.9	67	4
	Plasminogen	PLG	6156.1	1	0.8	0.8	1.0	68	4
	Pai I	SERPINE1	11.6	3	0.9	2.9	0.5	69	1
Lipid	Acyl-Coenzyme A oxidase 2, branched chain	ACOX2	122.2	1	0.8	0.9	0.9	70	4
metabolism	L-3-hydroxyacyl-Coenzyme A dehydrogenase, short chain	HADHSC	139.8	1	0.8	0.9	0.9	71	4
	Acyl-CoA synthetase long-chain family member 1	ACSL1	2800.8	1	0.8	0.9	0.9	72	4
	Acyl-Coenzyme A oxidase 1, palmitoyl	ACOX1	357.7	1	0.7	0.9	1.0	73	4
	Carnitine O-octanoyltransferase	CROT	10.3	1	0.7	0.8	1.1	74	4
	2,4-dienoyl CoA reductase 2, peroxisomal	DECR2	135.2	1	0.8	0.9	0.9	75	4
	Acetyl-Coenzyme A acyltransferase 2	ACAA2	1167.7	1	0.8	0.8	1.1	76	4
	Acetyl-Coenzyme A acetyltransferase 1	ACAT1	714.2	1	0.7	1.1	0.8	77	4
	Acyl-CoA synthetase long-chain family member 5	ACSL5	57.3	1	0.8	0.9	1.0	78	4
	Dodecenoyl-Coenzyme A delta isomerase	DCI	327.2	1	0.9	0.8	0.9	79	4
	Enoyl Coenzyme A hydratase, short chain, 1, mitochondrial	ECHS1	324.3	1	0.7	1.0	0.9	80	4
	Hydroxyacyl-Coenzyme A dehydrogenase, type II	HADH2	422.5	1	0.7	0.9	0.9	81	4
	Hydroxyacyl-Coenzyme A dehydrogenase, beta subunit	HADHB	398.8	1	0.8	0.9	1.1	82	4
	Lipase, hepatic	LIPC	561.5	1	0.5	1.5	0.9	83	4
	Hydroxyacyl-Coenzyme A dehydrogenase, alpha subunit	HADHA	103.2	1	0.9	0.9	1.0	84	4
	Palmitoyl-protein thioesterase 1	PPT1	82.1	1	1.0	0.8	0.8	85	4
	Fatty acid synthase	FASN	105.3	3	0.8	1.8	0.6	86	4
	Peroxisomal D3, D2-enoyl-CoA isomerase	PECI	292.8	1	0.8	0.8	0.8	87	4
	Acyl-CoA synthetase long-chain family member 4	ACSL4	5.8	2	1.1	1.4	2.3	88	4
Insignificant change: BHHADH, ACAA1, CPT1A, ACADM, ACACA, CPT2									
Steroid	Aldo-keto reductase family 1, member D1	AKR1D1	248.3	1	0.6	0.8	0.6	89	4
(or drug)	HMT1 hnRNP methyltransferase-like 2	HRMT1L2	6.6	1	0.8	1.0	0.8	90	4
metabolism	Hydroxysteroid (11-beta) dehydrogenase 1	HSD11B1	1064.6	1	0.7	0.8	0.8	91	4
	Hydroxysteroid (17-beta) dehydrogenase 4	HSD17B4	66.7	1	0.8	0.9	0.9	92	4
	Steroid-5-alpha-reductase, alpha polypeptide 1	SRD5A1	74.3	1	0.7	0.8	0.9	93	4
	UDP glycosyltransferase 2 family, polypeptide B7	UGT2B7	530.3	1	0.8	0.8	0.9	94	4
	Sulfotransferase family 1E, estrogen-preferring, member 1	SULT1E1	52.8	1	0.5	0.5	1.4	95	7
	Aldo-keto reductase family 1, member C4	AKR1C4	137.1	1	0.9	0.6	1.2	96	4
	Hydroxysteroid (17-beta) dehydrogenase 2	HSD17B2	496.9	1	1.0	0.8	1.1	97	4
	Sulfotransferase family, cytosolic, 2A, member 1	SULT2A1	689.0	1	0.9	0.8	0.9	98	4
	Hydroxysteroid (17-beta) dehydrogenase 8	HSD17B8	72.2	1	0.7	0.7	1.0	99	7
	Steroid sulfatase (microsomal), arylsulfatase C, isozyme S	STS	10.9	1	1.0	1.0	0.6	100	4
	Emopamil binding protein (sterol isomerase)	EBP	197.0	1	1.0	0.7	0.7	101	5
	Farnesyl-diphosphate farnesyltransferase 1	FDFT1	252.6	1	0.7	0.9	0.7	102	5
Insignificant change: HSD17B2, HSD3B1, LCM1, SULT2A1, HMGCR, DHCR7, CES2									
Bile acid	Sterol O-acyltransferase 1	SOAT1	9.1	1	1.0	0.8	0.9	103	5
metabolism	Alcohol dehydrogenase 1C (class I), gamma polypeptide	ADH1C	312.7	1	0.7	0.6	1.1	104	5
	Alcohol dehydrogenase, iron containing, 1	ADHFE1	112.7	1	0.7	0.7	0.8	105	5
	Cytochrome P450, family 7, subfamily A, polypeptide 1	CYP7A1	104.1	1	0.7	0.6	2.9	106	5
Prostanoid	Arachidonate 5-lipoxygenase-activating protein	ALOX5AP	7.9	2	1.7	0.7	1.0	107	3
	Leukotriene B4 receptor 2	LTBR2	2.5	1	1.1	0.7	1.0	108	3
Insignificant change: LTA4H, CYSLTR1, CYSLTR2, LTC4S, PPT1									
Aromatic	Dopa decarboxylase	DDC	51.4	1	1.0	0.8	0.8	109	4
amino acid	Monoamine oxidase B	MAOB	426.6	1	0.8	0.8	1.0	110	4
metabolism	Kynurenine 3-monoxygenase	KMO	51.7	1	1.0	0.7	1.0	111	4
	Kynureninase	KYNU	63.3	1	0.9	0.7	0.8	112	4
	Tyrosine aminotransferase	TAT	658.2	1	0.9	0.5	1.3	113	4
	GTP cyclohydrolase 1	GCH1	49.6	1	0.9	0.7	0.9	114	4
Insignificant change: HPD									
Sulfur-containing	MAT2	MAT2B	111.7	1	1.1	0.7	1.1	115	3
amino acid	Cystathionase (cystathionine gamma-lyase)	CTH	112.0	1	1.0	0.7	1.1	116	3
metabolism	Cystathionine-beta-synthase	CBS	350.4	1	1.1	0.7	0.9	117	3
	Betaine-homocysteine methyltransferase	BHMT	1032.1	1	1.0	0.7	1.0	118	3
	Methionine adenosyltransferase I, alpha	MAT1A	547.6	1	1.0	0.5	1.1	119	2
	Cysteine dioxygenase, type I	CDO1	84.4	1	0.9	0.7	1.0	120	1
	Glutamate-cysteine ligase, catalytic subunit	GCLC	175.9	1	1.1	0.6	1.1	121	1
	Glutathione S-transferase A1	GSTA1	2812.3	1	1.0	0.7	1.0	122	1
	Alanyl (membrane) aminopeptidase	ANPEP	501.9	1	0.9	0.8	0.9	124	5
	Bile acid Coenzyme A: amino acid N-acyltransferase	BAAT	284.4	1	1.0	0.6	1.0	125	5
	Glutathione synthetase	GSS	70.1	1	1.1	0.8	0.8	126	5
	Lactate dehydrogenase A	LDHA	716.3	1	0.8	0.7	1.0	127	5
	Mercaptopyruvate sulfurtransferase	MPST	684.3	1	0.9	0.7	0.9	128	5
	Serine dehydratase	SDS	440.4	1	0.9	0.4	1.0	129	5
	Methionine adenosyltransferase II, alpha	MAT2A	122.9	1	1.0	0.9	0.8	130	5
Insignificant change: GGT1, GSR, MTR, DNMT1, CSAD, GCLM, LDHB									

Energy source	Phosphoenolpyruvate carboxykinase 2 (mitochondrial)	PCK2	791.8	1	0.8	0.9	1.0	131	4
amino acid	Alanine-glyoxylate aminotransferase	AGXT	3352.3	1	0.7	0.9	1.0	132	4
metabolism	Alanine-glyoxylate aminotransferase 2	AGXT2	122.8	1	0.6	0.8	1.0	133	4
	Aldehyde dehydrogenase 2 family (mitochondrial)	ALDH2	2027.7	1	0.7	0.9	0.9	134	4
	Aldehyde dehydrogenase 9 family, member A1	ALDH9A1	160.9	1	0.8	0.8	0.9	135	4
	Pyruvate kinase, liver and RBC	PKLR	211.3	1	0.6	1.1	0.7	136	4
	Aldehyde dehydrogenase 3 family, member A2	ALDH3A2	237.4	1	0.9	0.8	1.1	137	4
	Phosphoenolpyruvate carboxykinase 1 (soluble)	PCK1	3166.8	1	1.0	0.5	1.2	138	4
	Dihydrolipoamide dehydrogenase	DLD	72.3	1	0.9	0.8	0.9	139	4
	Glutaminase 2 (liver, mitochondrial)	GLS2	133.8	1	0.8	0.7	1.0	140	4
	Glutamate-ammonia ligase	GLUL	6.2	1	1.0	0.6	1.2	141	4
	Glutamic-oxaloacetic transaminase 1, soluble	GOT1	388.2	1	0.9	0.7	0.8	142	4
	Glutamic-pyruvate transaminase	GPT	298.8	1	0.8	0.6	1.1	143	4
	Pyruvate carboxylase	PC	27.8	1	0.7	0.7	0.9	144	4
	Phosphoglucosmutase 1	PGM1	218.8	1	0.8	0.8	1.0	145	4
	Pyruvate dehydrogenase kinase, isoenzyme 2	PDK2	24.0	1	0.9	0.8	0.9	146	7
	Pyruvate dehydrogenase kinase, isoenzyme 4	PDK4	85.6	1	0.7	0.5	1.4	147	7
	Fumarylacetoacetate hydrolase	FAH	85.8	1	0.9	0.9	0.8	148	4
	Malic enzyme 1, NADP (+)-dependent, cytosolic	ME1	4.9	2	1.0	1.0	1.3	149	4
	Insignificant change: ALDOA, ASNS, GOT2, MGC33309, PDHB, PDK1								
Glucose	Phosphorylase, glycogen; liver	PYGL	37.2	1	0.8	1.0	1.0	150	4
metabolism	Aldolase B, fructose-bisphosphate	ALDOB	13141.8	1	0.7	0.8	0.9	151	4
	Hexokinase 3 (white cell)	HK3	3.3	1	1.2	0.6	0.9	152	4
	Glycogen synthase 2 (liver)	GS2	276.6	1	0.9	0.6	0.9	153	7
	Sterol regulatory element binding transcription factor 1	SREBF1	10.6	2	1.1	1.2	0.9	154	4
	Glycerol-3-phosphate dehydrogenase 1 (soluble)	GPD1	130.8	1	0.8	1.0	0.8	155	4
	Ketohexokinase (fructokinase)	KHK	411.8	1	0.7	1.0	0.8	156	4
	Glucokinase (hexokinase 4) regulator	GCKR	204.8	1	0.7	1.0	0.8	157	4
	Aldolase C, fructose-bisphosphate	ALDOC	36.8	1	0.7	0.9	0.6	158	4
	Insignificant change: GCK, PFKFB1, G6PC, HK2								
Urea cycle	Carbamoyl-phosphate synthetase 1, mitochondrial	CPS1	1230.8	1	0.7	0.9	0.9	159	7
	Ornithine aminotransferase (gyrate atrophy)	OAT	94.8	1	0.5	0.6	1.6	160	7
	Insignificant change: OTC								

Liver biopsy samples were analyzed by RT-PCR using a TaqMan® Gene Expression Assay probe, as described in the Materials and Methods. The genes were selected from DNA microarray data from a rat fibrosis model presented in a previous paper 3. Genes in the ECM category (or other HSC markers) were selected as marker genes for HSC. Genes in the inflammation category were selected as markers of inflammatory cells in liver. Other genes were selected as markers of hepatocytes. Expression profiles of marker genes that showed statistically significant changes during fibrosis by a Kruskal-Wallis test are listed. Genes that showed statistically insignificant changes in expression are listed with the gene name only in Table 1. Columns from left to right indicate functional category, gene description, gene name, expression intensity at F1 stage, group classification (expression of marker genes in group 1 decreased almost linearly along with the F stages, expression of marker genes in group 2 increased almost linearly along with the F stages, and expression of marker genes in group 3 had a peak in the middle of the F stages), expression ratio between neighbouring F stages (a decreased ratio is shown in light gray and an increased ratio in deep gray), serial gene number and types of samples, which indicates the number of biopsy specimens, as shown in the Table 2. Actual alteration behavior of some characteristic genes are shown in Figure 1.

Table 2 Types of samples indicating the number of biopsy specimens

Type of sample	Number of samples			
	F1	F2	F3	F4
1	25	13	14	9
2	23	13	14	8
3	22	12	14	8
4	12	6	9	4
5	7	4	5	4
6	6	4	4	3
7	5	4	5	5

Quantitative limitation of biopsy specimens resulted in a difference in sample number for each probe, according to the interest in the particular gene.

transition to an adjacent F stage are similar to the selected genes in Table 1. An additional four marker genes selected in the *t* test analysis were added to the genes in Table 1. Over half of the selected marker genes from the DNA microarray data from the animal model showed statistically significant changes in the human samples, showing the effectiveness of the animal model for selection of genes

of significance in humans.

Genes showing changes in expression were roughly divided into three groups: genes in group 1 showed an almost linear decrease in expression along with an increase in F stage; genes in group 2 showed an almost linear increase with an increase in F stage; and genes in group 3 showed a peak in the middle of the F stage scale. Almost all genes in one category belonged to one or two groups, suggesting that genes in one category showed similar changes in expression during progression of fibrosis. The expression ratio between F stages is also shown in Table 1, with the peak ratio shown in bold. The peak for almost all genes in a given category occurred at the transition to the same F stage, again suggesting that genes in one category underwent changes in expression under similar mechanistic control.

Expression changes in gene clusters in the early phase (F1 to F2) of fibrosis

The functional categories showing a peak change in expression ratio in the early phase of fibrosis were inflammation, ECM, blood coagulation, lipid metabolism, half of the

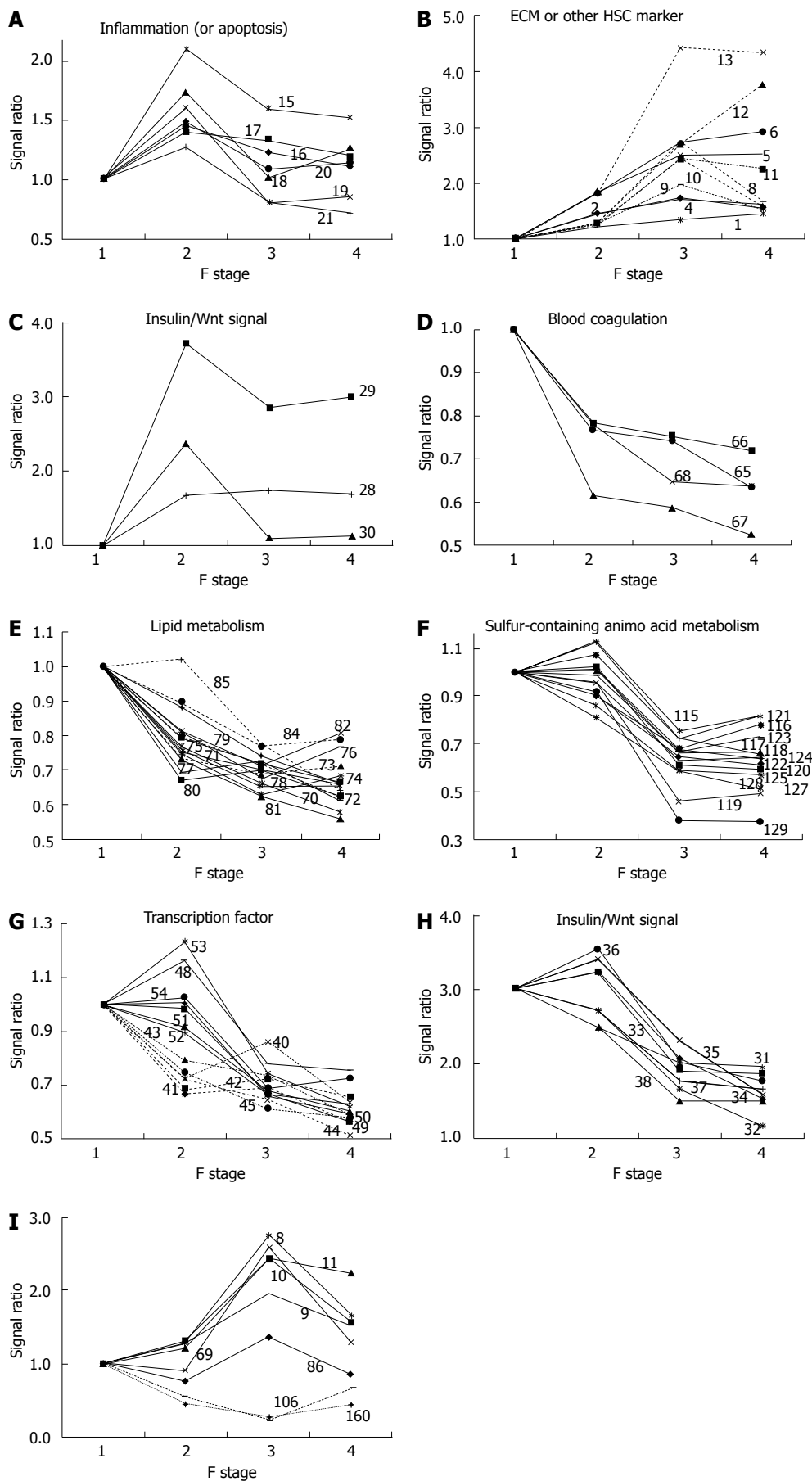


Figure 1 Clusters of genes in functional categories. The characteristic behavior of gene clusters for each functional category is shown separately in Figure 1A-I. The numbers in each figure refer to the serial number of genes in Table 1. The expression ratio relative to the F1 stage is plotted in each graph. **A:** Inflammation gene cluster with a peak at F2; **B:** ECM gene cluster showing increased expression along with fibrosis progression; **C:** Insulin/Wnt signaling gene cluster with increased expression in the early phase of fibrosis; **D:** Blood coagulation factor gene cluster showing decreased expression along with fibrosis progression; **E:** Lipid metabolism gene cluster showing decreased expression along with fibrosis progression; **F:** Sulfur-containing amino acid metabolism gene cluster showing a synchronous decrease in expression from F2 to F3; **G:** Two transcription factor gene clusters showing sequential decreases in expression; **H:** Insulin/Wnt signaling gene cluster showing decreased expression from F2 to F3; **I:** A gene cluster showing a peak or bottom at F3.

genes in steroid metabolism, half of those in energy source amino AA metabolism, and half of transcription factors.

Expression of marker genes in the inflammation and ECM categories started to increase in the early phase. Expression

Table 3 Classification of gene clusters based on serial alteration of gene expression along with the fibrosis progression

F1-F2	F2-F3	F3-F4
Inflammation		
Wound-healing (ECM)		
Blood coagulation		
Transcription factors (cluster 1)	Transcription factors (cluster 2)	
	Insulin/Wnt signal	
Lipid metabolism		
Steroid metabolism (cluster 1)	Steroid metabolism (cluster 1)	
	Bile acid metabolism	
Energy source AA metabolism (cluster 1)	Energy source AA metabolism (cluster 1)	
	Aromatic AA metabolism	
	Sulfur-containing AA metabolism	
	Glucose metabolism (cluster 1)	Glucose metabolism (cluster 2)

Gene clusters are indicated under the name of gene category. The pattern of serial alteration for gene cluster was determined based on the maximum changing point of the expression ratio shown in Table 1.

of marker genes related to inflammation, such as LYZ, GZMB, IL1B, TNF and TGFB1, occurred in clusters and reached a peak at fibrotic stage F2, as shown in Figure 1A, suggesting that inflammatory events are particularly active at the F2 stage. However, histological classification of inflammatory activity shows a tendency for an increase in inflammation that is correlated with an increase in F stage; therefore, the conclusion regarding inflammatory events based on expression of marker genes appears to differ from that based on histological classification. Gene expression in the ECM category also increased until F3 or F4, as shown in Figure 1B; expression of such genes might indicate an inflammatory response for wound healing. Increased expression of some clustered genes related to the cell cycle, CCND1, FOXM1 and GJA1 (Connexin 43), was also found at an early stage, as shown in Figure 1C, and might reflect a response to hepatic cell injury.

Almost all other genes were linearly down-regulated. Several genes related to blood coagulation, i.e. F10, AGT, FGA and PLG, were down-regulated as a cluster in the early phase, as shown in Figure 1D; this down-regulation may be linked to prolongation of the blood coagulation time in cirrhosis. An early response of many clustered genes associated with lipid metabolism was also found, as shown in Figure 1E. Expression of these genes decreased consecutively during fibrosis and the early response of genes affecting lipid metabolism is of interest.

Expression changes in gene clusters in the middle phase (F2 to F3) of fibrosis

The peak change in the expression ratio of marker genes in metabolism of sulfur-containing AA and aromatic AA was delayed, compared to genes associated with other kinds of metabolism. Marker genes for sulfur-containing AA metabolism decreased remarkably as a cluster in the phase from F2 to F3, as shown in Figure 1F. Decreased expression of marker genes for steroid metabolism, energy

source AA, and transcription factors were separable into two groups: early-response and middle-response genes.

All the down-regulated transcription factors, including TCF1 (HNF-1), HNF4A (HNF-4), CEBPA (C/EBP alpha), CEBPB (C/EBP beta), PPARG (PPAR alpha), RXRA (RXR alpha), NR1H3 (LXRA), NR1H2 (LXRB), NR1H4 (FXR), USF-1, USF-2, and NR0B2 are important in hepatic metabolism and other regulatory mechanisms. Alteration of expression of these genes might be related to abnormal expression of metabolic enzymes. Two clusters of transcription factors were clearly distinguishable based on their expression pattern, as shown in Figure 1G: the first cluster, including HNF-4, C/EBPA, RXR, TCF1 (HNF1), PPARG, and NR0B2, which showed altered expression in the early phase, might influence expression of the second cluster, including CEBPB, NR1H2(LXRB), NR1H3(LXRA), NR1H4(FXR), ESRRA and USF2, which showed altered expression in the middle phase of fibrosis.

A cluster of genes associated with insulin/Wnt signaling were down-regulated, as shown in Figure 1H, suggesting a common regulatory mechanism. These expression changes are likely to be related to changes in expression of transcription factors and genes in metabolic networks. The down-regulated genes included GSK3B and CTNNB1 (catenin beta 1), which participates in Wg/Wnt signaling for regulation of cell proliferation and differentiation^[4]; GJA1 (connexin 43), which forms gap junctions and is regulated by Wg/Wnt signaling^[5]; and FOXM1, which is associated with cell proliferation^[6] and liver regeneration^[7]. All of these genes had peak expression at F2 in a clustered manner, as shown in Figure 1C and G. Enhancement of cell proliferation for wound healing might be linked with a peak of inflammation at F2, and expression of genes such as CCND1, GJA1 and FOXM1 in the downstream part of the insulin/Wnt pathway were altered ahead of genes involved in insulin/Wnt signaling. The relationship between these genes requires further study.

Expression changes in gene clusters in the late phase (F3 to F4) of fibrosis

Few genes showed altered expression in the late phase of fibrosis, but a cluster of genes in the glucose metabolism category showed decreased expression. It was also of interest that expression of several genes reversed direction or abruptly altered in the late phase, as shown in Figure 1I. These results suggest different biological changes from the start of the late stage in the transition from F3 to F4.

Serial expression changes for the functional categories are summarized in Table 3 and these data indicate associations between clustered genes in one category and inter-category relationships.

Down-regulated individual marker genes (group 1)

Regeneration of hepatic cells is suppressed during fibrosis and such suppression is thought to then cause further fibrosis. IGF1, GHR and IL6R (inflammation) support the regeneration of hepatocytes and down-regulation of the expression of these genes may be linked directly to formation of fibrosis. Down-regulation of PON1, which associates with HDL (high-density lipoprotein)

and regulates the cellular redox state, and PPT, which is known as a lysosomal hydrolase of long chain fatty acyl CoA and has a role in maintenance of synaptic function, may be related to mitochondrial damage, as we and others have suggested^[3,8,9]. RGN, a calcium-binding protein that plays a pivotal role in maintaining cell homeostasis and function, was also down-regulated. Down-regulation of these genes may impair liver function. TRIB3 inhibits Akt/PKB activation by insulin^[10], and this gene was suddenly down-regulated from the F3 to F4 phase, suggesting new conditions in the insulin signaling pathway in the transition from F3 to F4.

Up-regulated individual marker genes (group 2)

Most inflammatory marker genes showed peak expression in the middle phase of fibrosis, as shown in Figure 1A, but CMA1 (chymase 1), which is produced by mast cells, underwent a linear increase in expression with progression of fibrosis. This is of note, since chymase has been reported to be involved in chronic hepatic fibrosis in autoimmune hepatitis and primary biliary cirrhosis^[11], and mast cells may have a special role in fibrogenesis. The role of mast cells in chronic inflammation, however, deserves further study, because of the shortage of determined sample and low expression of CMA1.

Individual marker genes with peak expression in the middle phase of fibrosis (group 3)

The only hepatic stellate cell (HSC)-specific marker gene to show peak expression at the F2 stage was PRNP, which is reported to be a marker for the early phase of HSC activation. The amino acid transporters SLC38A2 (ATA2), SLC6A6 (TAUT) and SLC7A1 (CAT-1) showed peak expression at F2, which may also suggest enhancement of cell proliferation at this stage of fibrosis. Increased expression of SLC38A2, which preferentially transports alanine, has been reported in regeneration of hepatocytes^[12], since hepatocytes require alanine as an energy source^[13]. Based on our results, down-regulation of SLC38A2 in the late phase of fibrosis suggests that utilization of alanine as an energy source decreases at this stage of fibrosis. Up-regulation of SLC6A6, a taurine transporter, in the early phase of fibrosis can be understood as protective behavior against injury of hepatocytes^[14]. FASN, a marker gene for fatty acid metabolism, showed peak expression at the F3 stage of fibrosis. We have observed suppression of biosynthesis and degradation of fatty acids in the liver in a CCl₄-induced cirrhotic rat model, and clinical results show temporal enhancement of fatty acid biosynthesis before subsequent suppression in an advanced phase of fibrosis; this may be a compensative action related to suppression of other forms of energy metabolism in fibrosis, as we have previously suggested^[5].

Candidate marker genes for diagnosis of fibrosis and discrimination of fibrotic stages

Diagnosis in the early stage of fibrosis may be important for monitoring progression of fibrosis and hepatic biological changes. Candidate marker genes at each step of fibrosis were selected based on an expression change

ratio > 1.5 and an intensity > 10. Up-regulation of LYZ and down-regulation of FGA, OAT and AGXT2 were noteworthy in the transition from F1 to F2, and up-regulation of genes in the ECM category and down-regulation of genes in metabolism of energy source AA, aromatic AA, steroids and sulfur-containing AA occurred in the transition from F2 to F3. Diagnosis of the late stage of fibrosis (the transition from F3 to F4) is important because of the risk of tumorigenesis. Some genes showed a reversal of expression in the F3 to F4 transition, suggesting that biological changes in the stage from F3 to F4 are qualitatively different from those at earlier stages. Remarkable down-regulation of TRIB3, which inhibits insulin signaling and NFκB signaling, was noteworthy in the F3 to F4 transition. The reversal in expression of FASN in this phase may indicate changes in lipid metabolism and that of OAT indicates changes in ornithine metabolism or the urea cycle. The early increase in collagen expression began to decline in the F3 to F4 stage and the similar decline of SERPINE1 (Pai1) expression may reflect increased fibrolysis. Consecutive analysis of marker molecules in plasma will be important for monitoring progression of fibrosis at each step, and the data in this paper provides useful information for the selection of serum markers and interpretation of changes in the levels of these markers.

Coordinated regulation of functional categories

All genes in Figure 1 were subjected to hierarchical clustering analysis. Statistical clustering of the expression ratio between neighboring F stages for the genes in Table 1 was combined with functional categories, as shown in Figure 2. Functional categories clearly corresponded to the statistical clusters, suggesting coordinated regulation of genes in one functional category. Overlap of functional categories in statistical clusters suggested that these categories might be regulated by correlated mechanisms. In contrast, separation of members of a category into multiple positions of a statistical cluster suggests that the functional category may be divided into subgroups with respect to regulation.

DISCUSSION

Biological interpretation of changes in gene expression

Changes in expression of hepatic cell-specific marker genes reflects biological changes in the progression of hepatic fibrosis. Shimizu *et al.*^[15] reported co-localization of chymase with fibrotic tissue, and we have reported increased expression of marker genes for mast cells, including chymase, in progression of fibrosis in a DNM-induced rat fibrosis model. The results reported here also show correlation of the expression of chymase with the stage of fibrosis. Therefore, these results suggest that a certain cell population producing chymase has an important role in the pathogenesis of fibrosis.

Many marker genes related to inflammation showed peak expression at F2. Inflammation has been reported to induce activation of HSCs^[16], and we have shown a peak in activated inflammatory cells in a DMN-induced fibrosis model. These results suggest that a temporal

of HNF-4 in human cirrhosis^[21] and of PPARs in hepatitis C virus genotype 3^[22] have been reported. C/EBP alpha and C/EBP beta regulate proliferation of hepatocytes^[23] and glucose and lipid homeostasis^[24-27], and HNF-1 and HNF-4 broadly regulate hepatic functions such as carbohydrate metabolism^[28], lipid metabolism^[29], bile acid metabolism and HDL-cholesterol metabolism^[30]; expression of HNF-1 is also regulated by HNF-4^[31]. USF1 and USF2 have been reported as glucose signals^[32,33], and the USF1 and USF2 homodimers and the USF1-USF2 heterodimer regulate expression of liver-specific genes such as apolipoprotein A2 and pyruvate kinase. HNF-4 and USF2a bind to the enhancer sequence cooperatively^[34]. HNF-4 also regulates PPAR alpha^[35], which in turn regulates glucose^[36], lipid^[37] and cholesterol metabolism^[38]. RXR alpha regulates lipid, bile acid and cholesterol homeostasis^[39], and LXR alpha, LXR beta and FXR are associated with lipid^[40,41], bile acid and cholesterol homeostasis^[40,42]. RXR and FXR form heterodimers with other transcription factors, including other members of the same family or with PPAR alpha^[40,43,44]. NR0B2 (SHP) regulates cholesterol metabolism^[45], glucose metabolism^[45] and bile acid synthesis^[46], and interacts with LXR^[47]. AKT1 and AKT2 are important kinases in the pathway of insulin regulation of glucose homeostasis^[48] and in fatty acid synthesis^[49]. Hence, the altered expression of these transcription factors may relate to altered expression of metabolic enzymes in glucose and lipid metabolism in the fibrotic liver of hepatitis C patients.

The continuous increase in expression of cyclin D1 (CCND1) correlated with F stage, as shown in Figure 1C, and appears to be important for hepatic tumorigenesis. The association of Wnt signaling^[50,51] and cyclin D^[52] with tumorigenesis is well known. Catenin beta 1 (CTNNB1) regulates cyclin D expression^[53] and is itself regulated through phosphorylation by GSK3B^[54] or other kinases^[55]. Down-regulation of expression of CTNNB1 occurred in advanced fibrotic stages, as shown in Figure 1H, and beta interacting protein 1 (CTNNBIP1), which is a negative regulator of CTNNB1, was also down-regulated, as also shown in Figure 1H. From this perspective, it was interesting that alterations in expression of genes associated with insulin signaling, including GSK3B, CTNNB1, CTNNBIP1 and downstream genes such as CCND1 and GJA1 (connexin 43), were clustered, as shown in Figures 1C and H. Suppression of insulin signaling has been reported in cirrhosis^[56]; however, the response of downstream genes in this pathway was inconsistent with suppression of the insulin signal. Therefore, further studies will be necessary to clarify whether this inconsistency arises from differences between expression levels and the activity of the protein products of these genes, or if another signal^[57] is involved in the Wnt and insulin signaling pathways.

The bioSpace Explorer is a system for analysis of DNA microarray data that may allow an understanding of the molecular relationships underlying the above results. The network of genes from previous reports revealed integral relationships among insulin/Wnt signal, CEBPA, PPARG, RXRA, glucose metabolism, lipid synthesis and lipid metabolism. An initial version of bioSpace Explorer was constructed based on molecular networks related to lipid metabolism, with entries showing relationships between

molecules *via* a line between the molecules. This bird's eye view of the pathway including lipid metabolism with the input PCR data is illustrated in Figure 3; molecules related to inflammation were up-regulated and many genes related to lipid metabolism were down-regulated.

Hypothetical causes of biological changes in progression of fibrosis

Biological changes in fibrosis can be summarized as follows. Initially, Kupffer cells or other inflammatory cells are activated in the transition from F1 to F2. This event immediately influences production of ECM and cell cycle genes for wound-healing^[58]. Blood coagulation is quickly suppressed in moving from F1 to F2, as shown by down-regulation of coagulation factor genes and up-regulation of the inhibitor, PAI-1. Several transcription factor genes are also immediately influenced, probably due to inflammation, as shown by the down-regulation of CEBPA, HNF4A, TCF1 and NR0B2. Expression of many genes associated with lipid metabolism also changed quickly in the transition from F1 to F2. Down-regulation of these genes may be controlled by down-regulation of the transcription factors, especially RXRA, PPARG, LXRs and FXR. Some genes related to steroid metabolism also responded quickly for control of inflammation.

Expression of genes associated with sulfur-containing AA metabolism and aromatic AA metabolism changed simultaneously in the transition from F2 to F3. The first type of metabolism relates to the redox state^[59] and the second is associated with production of active metabolites such as catecholamines and serotonin. Such important biological states are controlled to maintain homeostasis through several mechanisms^[60] and this may explain the delayed change in expression of these genes. The expression of many genes related to intracellular signaling, including insulin/Wnt signaling, also changed simultaneously in the transition from F2 to F3. This delayed change may also reflect compensative action for hepatic cellular defects on metabolism for energy supply and/or hepatic cellular proliferation. The main molecules in fibrosis, such as collagens, increase in expression from F2 to F3 and cause development of fibrosis through activation of HSCs through a stimulatory cycle involving inflammatory cells, HSCs and hepatocytes, as described previously^[6].

Some quantitative biological changes started in the transition from F3 to F4. Down-regulation of genes associated with sugar metabolism and fatty acid synthesis at this stage might induce persistent defects in energy storage and supply to the liver. The liver transits into an inescapable negative cycle between this defect in the hepatic energy state and mitochondrial damage in cirrhosis. This negative cycle will be discussed in a future paper describing DNA microarray analysis of an animal model of cirrhosis.

Coordinated regulation of functional categories

Statistical cluster analysis showed coordinated regulation of functional categories in liver fibrosis. These regulatory mechanisms can be examined prospectively using bioSpace Explorer, and these results will be discussed in a future paper. In the current work, the clinical gene expression profiles assembled using RT-PCR, using genes

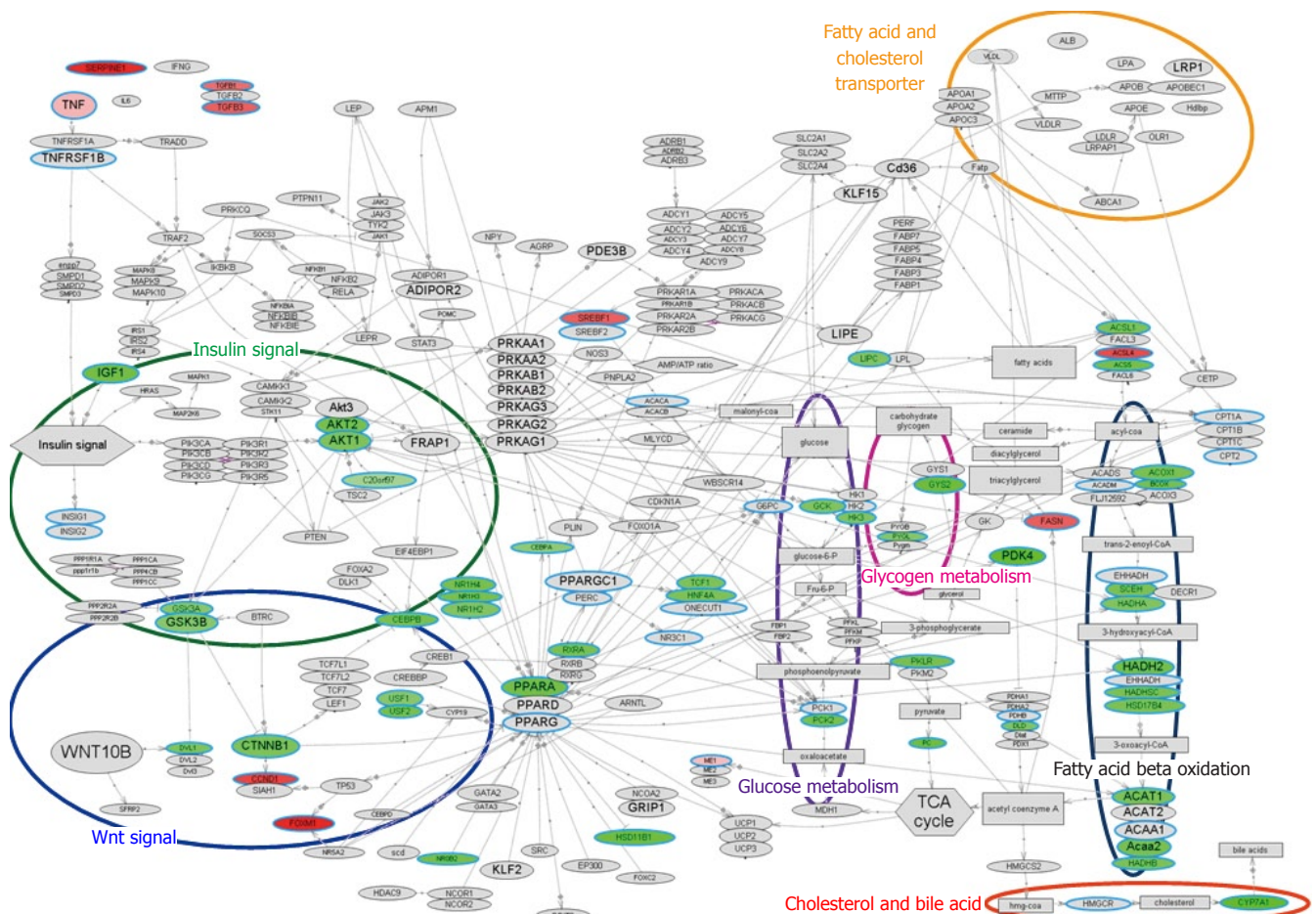


Figure 3 Molecular network associated with lipid metabolism. Gene expression changes in pathways related to lipid metabolism, illustrated with bioSpace Explorer (a system for analysis of DNA microarray data for lipid metabolism; Pharma Frontier Co. Ltd / World Fusion Co. Ltd.; see texts for details). The bird's-eye view of the lipid metabolism is displayed. The up-regulated and down-regulated gene expression ratios at F3 vs F1 in Table 1 are displayed in red and green, respectively, with the color gradation proportional to the ratio. Genes in Table 1 that did not show a statistically significant change in expression are indicated with a blue circle with gray background. An entry with a gray background only indicates no input data. Most of the entity names in Figure 2 are the same as the gene name in Table 1, but the names "C20orf97", "SCEH", "Acaa2", and "ACSL5" in Figure 2 refer to "TRIB3", "ECHS1", "ACAA2", and "ACSL5", respectively, in Table 1. These differences are due to the software used in bioSpace Explorer.

originally selected based on DNA microarray data from an experimental animal model, provided an improved understanding of disease and suggested new methods of diagnosis. Therefore, statistical analysis and functional clustering or network analysis of the transcriptome, alone or in combination, can provide an overview of a dynamic biological system.

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COMMENTS

Background

Information from clinical specimens is very important, but there is a limitation in sample preparation. With DNA microarray, it is difficult to determine gene expression profile from a small amount of sample such as a clinical needle biopsy sample. RT-PCR with TaqMan probe can make it possible with high quality. We have to get maximum information with a minimum number of TaqMan probes

because the amount of samples is limited and probes are expensive. Selection of probes is a key factor. We extensively determined gene expression profiles from animal models of liver fibrosis with DNA microarray (WJG 2006; 12: 6473). The gene marker sets were arranged to show the change of gene expression of a molecular network or functionally clustered gene sets. In this paper, the selected gene marker sets effectively showed the dynamic behavior of global gene network change during liver fibrosis.

Research frontiers

Dynamic behavior of genome-wide genes expression is now measured with DNA microarray. This technology must be applied to clinical samples. Such information can greatly advance the study of disease pathogenesis, diagnosis and therapy. One problem is the interpretation of huge expression profile data from DNA microarray. Advanced technology of computational text-mining has recently shown the genome-wide molecular networks or functional molecular clusters. This genome-wide network is going to be applied to the analysis of DNA microarray data. When expression profile data are arranged as a change in their networks of functional clusters, these huge data are expected to suggest effectively the biological meaning in terms of broad aspects of research interest. Therefore, we are developing an analysis system genome-wide molecular network which was made possible by a combination system based on both computational and manual text-mining. This system was partially applied on the interpretation of gene expression profile in this paper. Prevention therapy for individual patients at an early stage is required because genomic polymorphism is going to reveal the personal risk of diseases. The biological background of progression to disease onset must be understood for development of prevention therapy. Prediction of liver cancer risk is going to be tried in the early

stage of liver fibrosis before cirrhosis. The accumulation process of hepatic stress which leads to onset of liver cancer has to be elucidated. For example, there is a question why BCAA (branched chain amino acids), which improves hepatic metabolism, reduces onset of liver cancer.

Innovations and breakthroughs

We have already prepared the gene marker sets which can show the change of molecular networks or functionally clustered genes by DNA microarray experiment on liver fibrosis of animal models. The gene marker sets were linked to biological events in each hepatic cell such as hepatocytes, immune cells and stellate cells. Application of gene marker sets and RT-PCR with TaqMan probe technology on clinical specimens successfully showed the serial change of gene expression in molecular network or functionally clustered gene sets in the progression of liver fibrosis. It was proved that network analysis is a powerful tool for biological research. Our sequential approach (animal model/DNA microarray → selection of appropriate gene marker sets in molecular networks → clinical samples/RT-PCR → analysis functionally clustered gene markers in molecular networks → analysis of the relation between molecular networks) can effectively advance clinical research. Serial change of clustered gene expression during liver fibrosis progression, which was made clear in this paper, will reveal the molecular mechanism of many symptoms before and after the onset of cirrhosis and liver cancer.

Application

Gene marker sets and RT-PCR on clinical specimens as well as analytical methods with genome-wide gene networks can be applied to get the information of dynamic biological progression on various diseases. Serial change of clustered gene expression during liver fibrosis progression can provide a method of diagnosis and therapy in liver fibrosis. For example, the question why BCAA, which improves hepatic metabolism, reduces onset of liver cancer, will be solved.

Terminology

Text-mining: all published information about molecular interaction and their function are collected and arranged to make new valuable information such as gene-wide molecular networks; TaqMan-PCR probe: Applied Biosystems offers more than 700 000 TaqMan® Gene Expression Assays, the most comprehensive set of pre-designed Real-Time PCR assays available. All TaqMan® Gene Expression Assays have been designed using validated bioinformatics pipeline of Applied Biosystems, and run with the same PCR protocol, eliminating the need for primer design or PCR optimization.

Peer review

This paper revealed that metabolic deficiency occurs before the onset of cirrhosis. It had already been found in animal models with hepatic toxic substances in the preceding paper. Metabolic deficiency in hepatitis which was caused by a virus was found to be the same as animal models in this paper. Gene marker sets, selected from analysis of animal models, and analysis methods using molecular networks can lead to success in finding the serial change of functionally clustered genes expression during liver fibrosis progression. A set of appropriate gene markers in each network was a key to analysis. The sequential approach (animal model/DNA microarray → appropriate gene marker sets in molecular networks → clinical samples/RT-PCR → analysis functionally clustered gene markers in molecular network → analysis of the relation between molecular networks) is useful to elucidate the molecular mechanism of disease.

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Pancreatic stellate cells promote proliferation and invasiveness of human pancreatic cancer cells *via* galectin-3

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Abstract

AIM: To investigate the role of pancreatic stellate cells (PSCs) and galectin-3 (GAL-3) in the proliferation and infiltration of pancreatic cancer cell line SW1990.

METHODS: Human pancreatic cancer cell line SW1990 and PSCs were cultured *in vitro*. Supernatant fluid of cultured PSCs and SW1990 cells was collected. Expression of GAL-3 in SW1990 cells and PSCs was detected by ELISA, RT-PCR and Western blotting. Proliferation of cultured PSCs and SW1990 cells was measured by 3-(4, 5-methylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay and flow cytometry. Infiltration of SW1990 cells was detected by a cell infiltration kit.

RESULTS: SW1990 cells expressed GAL-3 and this was up-regulated by the supernatant fluid of cultured PSCs. PSCs did not express GAL-3. SW1990 cells stimulated proliferation of PSCs *via* GAL-3. GAL-3 antibody inhibited SW1990 cell proliferation, while the supernatant fluid of PSCs stimulated proliferation of SW1990 cells through interaction with GAL-3 protein. The supernatant fluid of PSCs enhanced the invasiveness of SW1990 cells through interaction with GAL-3.

CONCLUSION: GAL-3 and PSCs were involved in the proliferation and infiltration process of pancreatic cancer cells.

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Key words: Cell proliferation; Galectin-3; Infiltration; Desmoplastic reaction; Pancreatic cancer cell; Pancreatic stellate cell

INTRODUCTION

Tumor desmoplasia, a process in which fibrous tissue (e.g., collagen, fibronectin and laminin) infiltrates and envelops neoplasia, is one of the representative histopathological findings in ductal adenocarcinoma of the pancreas. The desmoplastic reaction may contribute to the rapid progression, early metastasis, and a limited response to chemotherapy and radiotherapy of pancreatic carcinoma^[1-5]. Some studies have confirmed that pancreatic cancer cells activate pancreatic stellate cells (PSCs) *via* transforming growth factor (TGF)- β and other cytokines^[6,7]. Although pancreatic carcinoma cells are able to produce the fibrotic extracellular matrix (ECM) that surrounds carcinoma, most studies have indicated that the fibrotic ECM is mainly produced and secreted by PSCs^[8].

Galectin-3 (GAL-3), is a member of the β -galactoside-binding protein family which recognizes the N-acetylglucosamine structure of various glycoconjugates^[9,10]. Studies on hepatic stellate cells (HSCs) have shown that GAL-3 stimulates HSC DNA synthesis in a dose-dependent manner, but no report on the effect of GAL-3 on PSCs has been published^[11]. Some studies have been carried out to evaluate the role of GAL-3 in carcinoma proliferation, infiltration and metastasis^[12-18], but few of these were on pancreatic cancer. Some immunohistochemical studies have reported that GAL-3 is expressed in pancreatic cancer^[19,20]. It has been confirmed that laminin, one important component of the fibrotic ECM that surrounds pancreatic cancer, can be recognized by GAL-3, so GAL-3 may play an important role in the progression of pancreatic cancer.

MATERIALS AND METHODS

Cell isolation and culture

SW1990 cells were cultured in 100 mL culture bottles containing Dulbecco's modified Eagle's medium (DMEM,

Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco) 100 U/mL penicillin and 100 U/mL streptomycin at 37°C in a 5% CO₂/air humidified atmosphere. PSCs were provided by our laboratory and were cultured in DMEM/F-12 supplemented with 10% FBS and antibiotics under the same conditions.

Collecting supernatants of cultured cells

SW1990 cells and PSCs cultured in 100 mL bottles were washed in PBS and then incubated with serum-free DMEM (5 mL/bottle) for 24 h. Supernatants of cells were collected and filtered under sterilized conditions to remove cell debris, and were stored at -80°C. The concentration of GAL-3 protein in these supernatants was detected by ELISA (Bender Systems).

RT-PCR

SW1990 cells were washed in PBS and incubated with serum-free DMEM for 24 h. The cells were divided into two groups, one was still incubated with serum-free medium, and the other was exposed to serum-free DMEM supplemented with 40% (v/v) supernatants of PSCs (SPSCs). Cells were harvested and counted 24 h later. Total RNA of 10⁶ cells in each group was extracted according to the manufacturer's instructions (TRIzol, Gibco-BRL, Rockville, MD, USA). The concentration and purity of RNA was determined by measuring the absorbance at 260 and 280 nm. After that, 1 µg total RNA in each group was reversed-transcribed to cDNA and amplified by RT-PCR (Gibco) according to the manufacturer's instructions. For detection of GAL-3 mRNA, the following oligonucleotide primers were used: 5'-ATGATGCGTTATCTGGGTCT-3' and 3'-TATTGGACGGAAACGGAC-5'. The amplification reaction involved denaturation at 94°C for 5 min followed by 35 cycles of denaturation at 94°C for 1 min, annealing for 1 min at 58°C, and extension for 1 min 30 s at 97°C. Expression of GAL-3 by PSCs was also detected by RT-PCR.

Western blotting

Two groups of SW1990 cells were prepared as mentioned for RT-PCR. SW1990 cells were centrifuged at 600 g for 10 min. The cell pellet was washed twice with ice-cold PBS, resuspended in 150 µL lysis buffer (1% Triton X-100 in 5 mmol/L Tris-HCl, pH 8.0, 15 mmol/L NaCl, 2 mmol/L PMSF). The fragmented cells were scraped and removed into a sterilized Eppendorf tube and conserved on ice for 20 min, then centrifuged at 12000 r/min for 20 min, and unresolved debris was discarded. Proteins were transferred onto PVDF membranes using the wet transfer technique, and the membranes were incubated overnight with monoclonal mouse anti-human-GAL-3 antibodies (R&D) diluted 1:200 in TBS. After 1 h incubation with horseradish peroxidase-labeled secondary antibodies (goat anti-mouse IgM diluted 1:10000 in TBS), GAL-3 was visualized with the ECL Western Blot Detection Kit (Gibco).

Cell proliferation test

Cell growth experiments were performed using the 3-(4, 5-methylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay and were reconfirmed by cell cycle analysis,

which was performed by flow cytometry. In the MTT assay, cells were seeded with medium that contained 10% FBS at a density of 6000 cells/well in 96-well plates, grown overnight, washed in PBS, and incubated with serum-free medium for 24 h. Cells were exposed to different concentration of SPSC (5%, 10%, 20% or 40%), supernatants of SW1990 cells (SSW; 5%, 10%, 20% or 40%), GAL-3 monoclonal antibody (GAL-3 MA; 10, 50 or 250 ng/mL, or 1.25 µg/mL), or recombinant human GAL-3 protein (5, 25, 125 or 625 ng/mL). Twenty-four hours later, MTT was added (50 µg/well) for 4 h. Formazan products were solubilized with DMSO, and the optical density was measured at 490 nm.

For flow cytometry, cells cultured in 100-mL culture bottles were washed in PBS and incubated with serum-free medium for 24 h. Cells were then exposed to 40% SPSC (for SW1990 cells), 40% SSW (for PSCs), 1 µg/mL GAL-3 MA or 100 ng/mL recombinant GAL-3 protein for 24 h. Cells were harvested and resuspended in fixation fluid at a density of 10⁶/mL, then 1800 µL trypsin solution was added to the fixation fluid, followed by 1500 µL RNase solution. Several minutes later, 1500 µL propidium iodide solution was added, and 15 min later, cells were filtered, and the cell cycle was detected by FACSCaliber (Becton Dickinson).

Cell invasion analysis

Invasion assays were carried out following the manufacturer's instructions of cell invasion assay kit (Chemicon International Inc., catalog: ECM550). For the invasion assay, we used a modified Boyden chamber. The chamber had two compartments divided by a polycarbonate filter (8 µm pore size), coated with a reconstituted basement membrane (ECMatrix solution). 3 × 10⁵ SW1990 cells were added to each upper compartment, and chemoattractant fluid was added to the lower compartment (Group A: control group, serum-free medium in the upper compartment and medium containing 2% FBS in the lower compartment; Group B, serum-free medium containing 1 µg/mL GAL-3 MA in the upper compartment and medium containing 2% FBS in the lower compartment; Group C, serum-free medium in the upper compartment and medium containing 2% FBS and 40% SPSC in the lower compartment; Group D, serum-free medium containing 1 µg/mL GAL-3 MA in the upper compartment and medium containing 2% FBS and 40% SPSC in the lower compartment). After 48 h incubation, non-invading cells were removed by cotton-tipped swabs and the filters were stained in the staining solution for 20 min and rinsed several times in water and air dried. Three filters were used per group. The number of invading cells was counted in 10 random high-powered fields per filter under a Zeiss microscope.

Statistical analysis

The data were expressed as mean ± SD and compared by ANOVA and the bivariate correlate test. *P* < 0.05 was considered statistically significant.

RESULTS

GAL-3 in SSWs and SPSCs

GAL-3 protein in SSWs and SPSCs was measured by

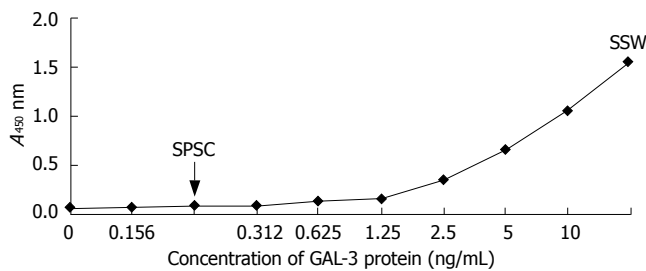


Figure 1 Concentration of GAL-3 in SPSC and SSW (ELISA).

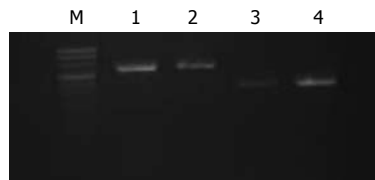


Figure 2 GAL-3 mRNA of SW1990 cells detected by RT-PCR. M: Marker; 1: GAPDH of control group; 2: GAPDH after stimulation with 40% SPSC; 3: GAL-3 of control group; 4: GAL-3 after stimulation with 40% SPSC.

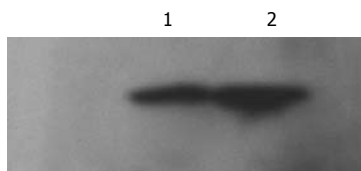


Figure 3 GAL-3 protein of SW1990 cells detected by Western blotting. 1: GAL-3 of control group; 2: GAL-3 after stimulation with 40% SPSC.

ELISA kit (Figure 1). The concentration of GAL-3 in SSWs was above the upper limit of the ELISA kit detection range (10 ng/mL), which meant that SW1990 cells could secrete relatively large amounts of GAL-3 into the extracellular fluid. There was little GAL-3 in SPSCs.

GAL-3 expression at the mRNA and protein level

Both RT-PCR (Figure 2) and Western blotting (Figure 3) confirmed that SW1990 cells expressed GAL-3, and the SPSCs up-regulated expression of GAL-3. PSCs showed no expression of GAL-3 according to RT-PCR and Western blotting.

PSC proliferation

According to MTT assay (Figure 4), GAL-3 antibody had no effect on the proliferation of PSCs. SSW stimulated PSC proliferation and this was partly inhibited by GAL-3 MA, which suggested that the stimulatory effect of SSW on PSC proliferation was partly mediated *via* GAL-3. This was confirmed by the effect of recombinant GAL-3 protein on PSC proliferation. According to the GAL-3 ELISA kit, the concentration of GAL-3 in SSW was > 10 ng/mL, and in the MTT assay, 5 ng/mL GAL-3 was sufficient to significantly stimulate PSC proliferation, which meant that GAL-3 secreted by pancreatic cells played a role in PSC proliferation in pancreatic cancer.

PSC proliferation detected by flow cytometry is show

Table 1 PSC cycle detected by flow cytometry (%)

Group	Medium	G0 + G1 fraction	S phase fraction	G2/M fraction	S + G2/M fraction
1 (Control)	Serum-free medium	89.81	1.94	8.25	10.19
2	Serum-free medium containing 40% SSW	89.63	3.23	7.14	10.37
3	Serum-free medium containing 40% SSW and 1 µg/mL GAL-3 antibody	89.42	2.70	7.88	10.58
4	Serum-free medium containing 100 ng/mL GAL-3 protein	87.04	3.68	9.28	12.96

Group 1: PSCs exposed to serum-free medium (control group); Group 2: PSCs exposed to serum-free medium containing 40% SSW; Group 3: PSCs exposed to serum-free medium containing 40% SSW and 1 µg/mL GAL-3 antibody; Group 4: PSCs exposed to serum-free medium containing 100 ng/mL GAL-3 protein.

in Table 1. The S-phase fraction of the control group was the lowest, that of group 4 was the highest, and group 2 had a higher S-phase fraction than group 3. This result was consistent with that of the MTT assay. PSCs are normal cells that grow very slowly, and have high demand on serum during culturing, so the S-phase fraction of all four groups was not very high.

Proliferation of SW1990 cells

According to MTT assay (Figure 5), GAL-3 MA inhibited proliferation of SW1990 cells, and this was positively related to antibody concentration, which suggested that GAL-3 protein was involved in the proliferation process of SW1990 cells. SW1990 cells may increase their proliferation by paracrine or autocrine GAL-3 protein. SPSC stimulated proliferation of SW1990 cells. After GAL-3 MA was added to serum-free DMEM containing 40% SPSC, absorbance declined with the increase in antibody concentration, but it was still higher than that in groups that only used GAL-3 MA. There was a significant correlation between the effect of GAL-3 MA and SPSC on absorbance, which suggested that the stimulatory effect of SPSC on proliferation of SW1990 cells was partly related to GAL-3.

SW1990 cell proliferation detected by flow cytometry is show in Table 2. SW1990 cells are quickly proliferating cancer cells, and the S-phase fraction may not properly reflect their proliferation. Therefore, we use the total S phase plus G2/M phase fraction to measure their proliferation. Compared with the control group, GAL-3 MA distinctly inhibited their proliferation, SPSC obviously stimulated proliferation, and GAL-3 MA partly inhibited the SPSC-induced stimulation. This result was consistent with the results of the MTT assay.

Invasion of SW1990 cells

The results of cell invasion are shown in Figures 6 and 7. GAL-3 MA had no effect on invasion of SW1990 cells. SPSC stimulated invasion of SW1990 cells and this was partly inhibited by GAL-3 MA, which suggested that the

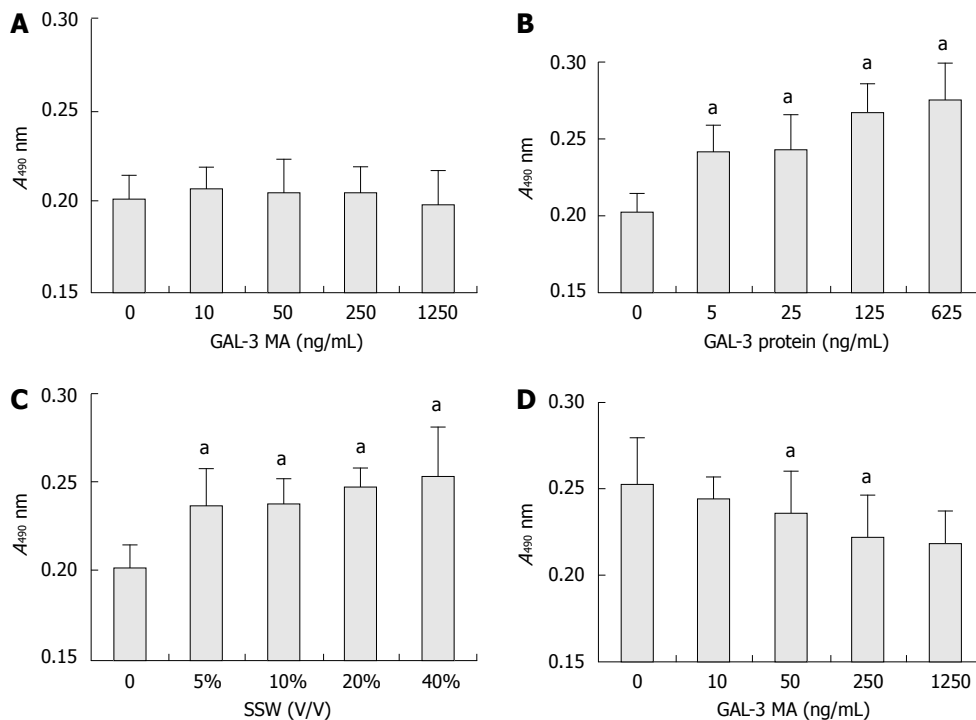


Figure 4 Proliferation of PSCs detected by MTT assay. (A) Absorbance at 490 nm after exposure to different concentrations of GAL-3 MA; (B) Absorbance at 490 nm after exposure to different concentrations of recombinant human GAL-3 protein; (C) Absorbance at 490 nm after exposure to different concentrations of SSW; (D) Absorbance at 490 nm after exposure to 40% SSW plus different concentrations of GAL-3 MA (^a*P* < 0.05).

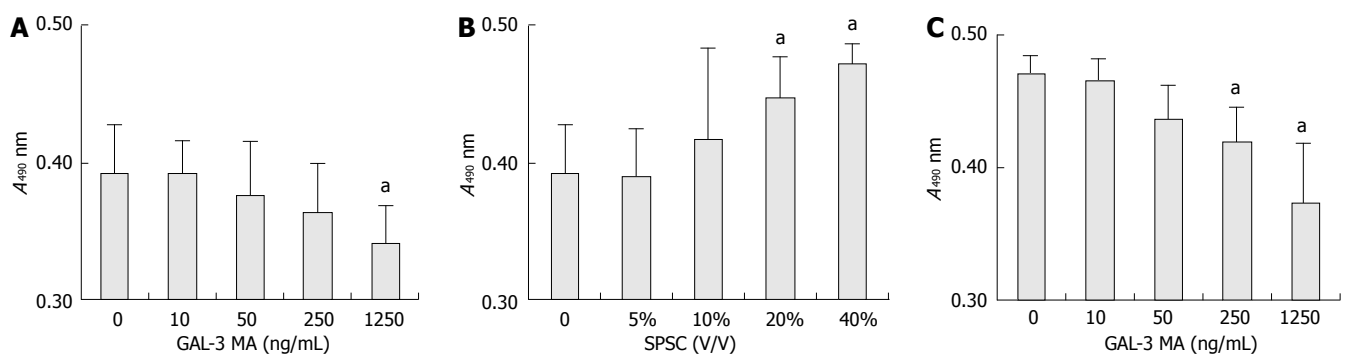


Figure 5 Proliferation of SW1990 cells detected by MTT assay. (A) Absorbance at 490 nm after exposure to different concentrations of GAL-3 MA; (B) Absorbance at 490 nm after exposure to different concentrations of SPSC; (C) Absorbance at 490 nm after exposure to 40% SPSC plus different concentrations of GAL-3 MA (^a*P* < 0.05).

Table 2 Proliferation of SW1990 cells detected by flow cytometry

Group	Medium	G0 + G1 fraction	S phase fraction	G2/M fraction	S + G2/M fraction
1 (control)	Serum-free DMEM	71.80%	16.76%	11.45%	28.20%
2	Serum-free DMEM containing 1 µg/mL GAL-3 MA	79.28%	13.87%	6.85%	20.72%
3	Serum-free DMEM containing 40% SPSC	62.51%	20.34%	17.15%	37.49%
4	Serum-free DMEM containing 40% SPSC and 1 µg/mL GAL-3 MA	67.91%	20.56%	11.53%	32.09%

Group 1: Sw1990 cells exposed to serum-free DMEM (control group); Group 2: SW1990 cells exposed to serum-free DMEM containing 1 µg/mL GAL-3 MA; Group 3: PSCs exposed to serum-free DMEM containing 40% SPSC; Group 4: PSCs exposed to serum-free DMEM containing 40% SPSC and 1 µg/mL GAL-3 MA.

stimulatory effect of SPSC on SW1990 cell invasion was partly mediated *via* GAL-3 expressed on the cells.

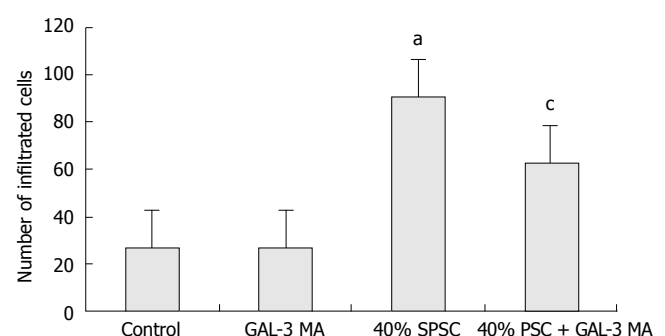


Figure 6 Number of infiltrating cells in each group. ^a*P* < 0.05 vs control; ^c*P* < 0.05 vs 40% SPSC.

DISCUSSION

Our study confirmed that human pancreatic cancer cell line SW1990 expressed and secreted GAL-3 protein, and that GAL-3 MA inhibited proliferation of SW1990 cells, which suggests that cancer cells such as SW1990 can stimulate their proliferation by autocrine or paracrine GAL-3.

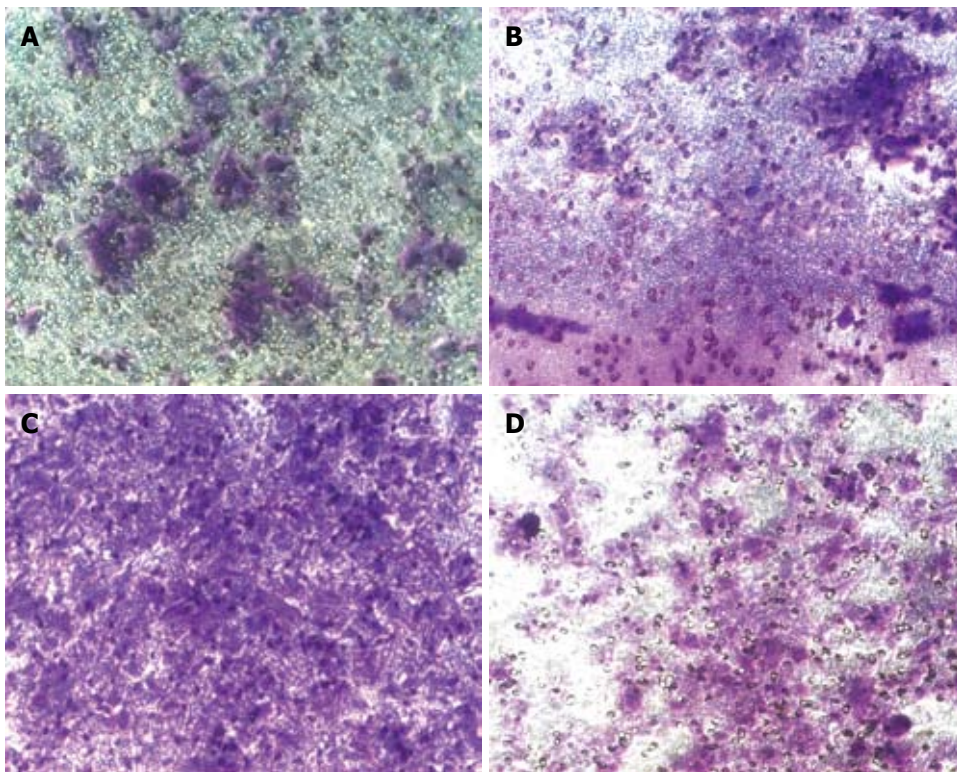


Figure 7 Infiltrating SW1990 cells. (A) Control group. (B) GAL-3 MA group. (C) SPSC group. (D) SPSC plus GAL-3 MA group. Compared with the control group, GAL-3 antibody had no significant effect on invasion of SW1990 cells. SPSC stimulated the invasion process of SW1990 cells, and this stimulation was partly inhibited by GAL-3 antibody.

However, more studies should be done to clarify this and to establish the signaling pathway through which GAL-3 stimulates proliferation of cancer cells.

Ductal adenocarcinoma of the pancreas is composed of infiltrating cancer cells surrounded by a predominant dense fibroblastic stroma. Previous studies have shown that the fibrotic ECM is mainly produced and secreted by PSCs, and pancreatic cancer cells activate PSCs *via* TGF- β and other cytokines such as basic fibroblast growth factor to increase production of ECM^[8]. Our study, including MTT assay and flow cytometry, confirmed that pancreatic cancer cells stimulated proliferation of PSCs *via* GAL-3, but further studies should be performed to elucidate the stimulation mechanism of GAL-3 on the proliferation of PSCs. Our study also confirmed that PSCs, through interaction with GAL-3, stimulated pancreatic cancer cells to proliferate, but the exact mechanism needs to be further investigated. Our study confirmed that pancreatic cancer cells and PSCs stimulated proliferation of each other. This may be one of the important reasons for the rapid progression of pancreatic cancer, and preventing this counter-stimulation may be very important in slowing the progression of the disease. GAL-3 is involved in the counter-stimulation between pancreatic cancer cells and PSCs, and this may help us to establish new methods for treating pancreatic cancer.

It has been shown by many studies that GAL-3 plays an important role in the interaction between cells and ECM, and this interaction is very important for carcinoma infiltration and metastasis. Activated PSCs can produce and secrete a lot of ECM, including collagen, fibronectin and laminin. Our infiltration study confirmed that the SPSC stimulated the infiltration of pancreatic cancer cells through interaction with GAL-3, which suggests that PSCs

play an important role in the early infiltration of pancreatic cancer. Since GAL-3 stimulated the proliferation of pancreatic cancer cells and PSCs, and was involved in neoplastic and ECM interaction, we suggest that it plays an important role in the infiltration and metastasis of pancreatic cancer.

COMMENTS

Background

Bachem *et al*^[9] discovered pancreatic stellate cells (PSCs) in 1998. After that, several studies showed that there are activated PSCs around pancreatic cancer tissues, pancreatic cancer cells can activate PSCs, and activated PSCs can produce a lot of extracellular matrix (ECM), which lead to the desmoplasia reaction in pancreatic cancer. Galectin-3 (GAL-3) is a member of the β -galactoside-binding protein family which recognizes the *N*-acetylglucosamine structure of various glycoconjugates. Many studies have shown that GAL-3 plays a role in carcinoma proliferation, infiltration and metastasis. One study has also shown that pancreatic cancer may express GAL-3, but the role of GAL-3 in pancreatic cancer has not been investigated.

Research frontiers

Previous studies have shown that pancreatic cancer cells can activate PSCs *via* cytokines such as TGF- β and platelet-derived growth factor, which are expressed by cancer cells, but few have discussed the role of activated PSCs in the progression of pancreatic cancer. Bachem *et al*^[9] have shown that GAL-1 can activate PSCs, and several others have shown that GAL-3 activates HSCs, but up till now, no study on the effect of GAL-3 on PSCs has been published.

Innovations and breakthroughs

This study showed that pancreatic cancer cells activated PSCs *via* GAL-3 expressed by cancer cells besides TGF- β and PDGF. It showed that activated PSCs promoted progress of pancreatic cancer by stimulating the proliferation and invasion of cancer cells. This study showed that there were complicated interactions between pancreatic cancer cells and PSCs. It also suggests that ECM and polysaccharides might play a role in the progress of pancreatic cancer, since GAL-3 is a lectin that recognizes the *N*-acetylglucosamine structure of various glycoconjugates.

Applications

This study provides a basis for future studies on the mechanisms of how GAL-3 activates PSCs, and how PSCs promote the proliferation and invasion of pancreatic cancer. It also provides a basis for future studies on treating pancreatic cancer through inhibition of GAL-3.

Terminology

SW1990 is a commonly used human pancreatic cancer cell line in pancreatic cancer research, which was first derived from spleen metastasis of a grade 2 ductal pancreatic cancer in the early 1980s. PSCs are vitamin A-storage cells that resemble HSCs in the healthy pancreas and comprise approximately 4% of all pancreatic cells; they show a periacinar distribution. GAL-3 is an endogenous β -galactoside-binding protein that is expressed widely in normal and neoplastic cells.

Peer review

This was a well-executed study. It explored the role of GAL-3 in the proliferation and invasion of pancreatic cancer cells *in vitro*, and showed that GAL-3 may play a role in the progression of pancreatic cancer.

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Probiotic intervention has strain-specific anti-inflammatory effects in healthy adults

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Author contributions: Kekkonen RA designed and coordinated the study, did the PBMC analysis participated in the interpretation of the results and wrote the paper; Lummela N participated in planning the study, was responsible for the study visits and created the database; Latvala S helped in the PBMC analysis; Karjalainen H and Tynkkynen S were responsible for the microbiological analysis; Kautiainen H and Järvenpää S participated in planning the study, carried out the statistical analysis, participated in the interpretation of the results and revised the manuscript; Julkunen I offered expertise in immunology and revised the manuscript; Vapaatalo H participated in planning the study and in the interpretation of the results, supervised the study and revised the manuscript; Korpela R initiated the study, participated in planning the study and the interpretation of the results, was responsible for the management of the study, and revised the manuscript.

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freudenreichii ssp. *shermanii* JS (PJS) or a placebo drink for 3 wk. Venous blood and saliva samples were taken at baseline and on d 1, 7 and 21. Fecal samples were collected at baseline and at the end of intervention.

RESULTS: The serum hsCRP expressed as the median AUC₀₋₂₁ (minus baseline) was 0.018 mg/L in the placebo group, -0.240 mg/L in the LGG group, 0.090 mg/L in the Bb12 group and -0.085 mg/L in the PJS group ($P = 0.014$). *In vitro* production of TNF- α from *in vitro* cultured peripheral blood mononuclear cells (PBMC) was significantly lower in subjects receiving LGG vs placebo. IL-2 production from PBMC in the Bb12 group was significantly lower compared with the other groups.

CONCLUSION: In conclusion, probiotic bacteria have strain-specific anti-inflammatory effects in healthy adults.

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Key words: Probiotic; Highly sensitive C-reactive protein; Cytokine; Inflammation; Immune response; Mononuclear cells

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Abstract

AIM: To evaluate the effects of three potentially anti-inflammatory probiotic bacteria from three different genera on immune variables in healthy adults in a clinical setting based on previous *in vitro* characterization of cytokine responses.

METHODS: A total of 62 volunteers participated in this randomized, double-blind and placebo-controlled parallel group intervention study. The volunteers were randomized to receive a milk-based drink containing either *Lactobacillus rhamnosus* GG (LGG), *Bifidobacterium animalis* ssp. *lactis* Bb12 (Bb12), or *Propionibacterium*

INTRODUCTION

Probiotics are defined as living microorganisms that have beneficial effects on human health^[1]. The immunomodulatory effects of probiotics have mostly been studied in certain disease conditions, such as allergies^[2] and inflammatory diseases^[3,4], though the general, healthy population mostly consumes probiotics. The immunomodulatory effects of probiotics in healthy populations have not been fully established and only a few randomized, double blind, placebo-controlled studies have addressed this question^[5-9]. Also, there are few studies where the effects of different

probiotic bacteria have been compared within the same clinical setting. Isolauri *et al*^[10] and Viljanen *et al*^[11] have compared the effects of two different probiotics or a probiotic mixture with placebo in allergic infants. Schifffrin *et al*^[12] and Gill *et al*^[13] evaluated the effects of two different probiotics in healthy adults, but these studies did not have a placebo group. Efforts trying to compare the *in vitro* results of one probiotic to its results in an *in vivo* setting are even more scarce and are at the moment limited to comparisons between *in vitro* and experimental animal studies^[14-16].

In our previous studies, we have characterized the capacity of potentially probiotic bacteria to induce cytokine production in human leukocyte cell culture and found that probiotic bacteria direct immune responses to either the Th1 type or the anti-inflammatory direction in a manner specific to the bacterial genera^[17]. Based on these findings we selected probiotic bacteria from three different genera for the present study and compared their effects on immune variables in healthy adults in a 3-wk intervention trial.

MATERIALS AND METHODS

Subjects

The subjects were healthy adults recruited by an advertisement in the Helsinki area. The inclusion criteria were to be healthy (no chronic illnesses), to exercise regularly (at least three times per week), and to not be participating in any other clinical trials. The exclusion criteria was comprised of milk allergies (due to the nature of the study products), use of antibiotics during the two months before the study, acute gastrointestinal disorders during the two months before the study, gastrointestinal diseases and related medications, pregnancy, and lactation. Before entering the study, the subjects gave their written informed consent. The study protocol was approved by the Ethics Committee of the Hospital District of Helsinki and Uusimaa.

A total of 68 subjects were recruited for the study. Six subjects withdrew from the study during the run-in period and were not included in the analysis. The mean age for the subjects was 44 years (range 23-58) and their mean BMI was 24 kg/m² (range 18-30). Of these 62 subjects (45 females, 17 males), one subject withdrew from the study due to a back injury after two study visits and one subject due to an antibiotic treatment after four study visits. These two subjects were included in the statistical analysis.

Study design and intervention

The study was a randomized, double-blind and placebo-controlled parallel group intervention study. Prior to the intervention period, there was a 3-wk run-in period during which no probiotic-containing products were allowed. Thereafter the subjects received either *Lactobacillus rhamnosus* GG (*n* = 13), *Bifidobacterium animalis* ssp. *lactis* Bb12 (*n* = 16), *Propionibacterium freudenreichii* ssp. *shermanii* JS (*n* = 17) or placebo (*n* = 16) drink for 3 wk. After the intervention period, subjects were followed up for 3 wk without any study drink. A list of probiotic-containing products was given to the subjects, and they were asked

not to consume any other probiotic-containing products at any time during the study.

Study products

The subjects were advised to consume a 250 mL milk-based fruit drink daily for 3 wk containing either: *L. rhamnosus* GG (ATCC 53103) (LGG) bacteria, on average 6.2×10^7 cfu/mL (daily dose of 1.6×10^{10} cfu); *B. animalis* ssp. *lactis* Bb12 (Bb12) bacteria, 1.4×10^8 cfu/mL (daily dose of 3.5×10^{10} cfu); *P. freudenreichii* ssp. *shermanii* JS (DSM 7067) (PJS) bacteria, 1.3×10^8 cfu/mL (daily dose of 3.3×10^{10} cfu); or a placebo drink without any probiotic bacteria. The subjects consumed the study drinks throughout the 3-wk intervention period after the baseline blood sampling. The amount of probiotic bacteria in the study drinks was measured right after packaging and after 3 wk. The appearance and taste of the study drinks were the same.

Blood samples

Venous blood samples from the antecubital vein were taken at baseline, on 1, 7 and 21 d, and after the 3-wk follow-up period after an overnight fast. The samples were taken into standard serum tubes and EDTA tubes, centrifuged, and the plasma/serum was collected and stored at -20°C for further analyses. Three EDTA tubes were used in the purification of PBMC.

Blood cells and immunoglobulins: Blood cells (leukocytes, monocytes, and lymphocytes) from all time points were determined using an electronic counter (Coulter MAXM Hematology Analyzer, Beckman Coulter, Fullerton, CA, USA). Immunoglobulins (IgA, IgG and IgM) from all time points were measured by immunoturbidimetric method with Tina-quant Roche/Hitachi System reagent using a Roche Hitachi 912 analyzer (Roche Diagnostics GmbH, Mannheim, Germany).

Highly sensitive C-reactive protein: Serum levels of C-reactive protein (CRP) were measured at all time points by a highly sensitive particle-enhanced immunoturbidimetric CRP (hsCRP) assay using a Tina-quant C-reactive protein (latex) high sensitive reagent and a Roche Hitachi 912 analyzer (Roche Diagnostics GmbH) with a detection limit of 0.04 mg/L.

Cytokine levels from serum: Baseline and 21 d cytokine levels (TNF- α , IL-6, IFN- γ and IL-10) in serum were determined using Quantikine HS, Human TNF- α /TNFSF1A (Catalog Number HSTA00D), IL-6 (HS600B), IFN- γ (DIF50) and IL-10 (HS100B) immunoassays purchased from R&D Systems (Minneapolis MN, USA). These assays were carried out according to the manufacturer's instructions. The detection limit was 0.5 pg/mL for TNF- α , 0.16 pg/mL for IL-6, 15.6 pg/mL for IFN- γ and 0.78 pg/mL for IL-10. For TNF- α , 94% of the samples were over the detection limit, and for IL-6, 89%. For statistical analyses, a detection limit divided by two was given as a value for those samples under the detection limit. None of the IFN- γ samples and only 39%

of the IL-10 samples was over the detection limit and were therefore not further analyzed.

PBMC cell culture

Purification: Human PBMC were purified by density gradient centrifugation over a Ficoll-Paque gradient (Amersham-Pharmacia Biotech, Uppsala, Sweden), as described previously^[18], from freshly collected EDTA blood on the study days (baseline, d 1, 7 and 21 wk and 3 wk after intervention). After washing, the cells were resuspended in RPMI 1640 medium (Sigma, USA) containing 10% heat-inactivated fetal calf serum (FCS) (Integro, Zaandam, Holland) and supplemented with 2 mmol/L L-glutamine (Sigma), 100 U/mL penicillin and 100 µg/mL streptomycin (Gibco BRL, Paisley, Scotland). In stimulation experiments, purified leukocytes (2×10^6 cells/mL) were incubated with stimulants in a final volume of one ml in 24-well plates (Nunc, Roskilde, Denmark) for 24 h in 5% CO₂ at 37°C.

Stimulations: During the stimulation experiments, the PBMC were maintained in RPMI-1640 medium containing 10% FCS. PBMC were left unstimulated or were stimulated with one of three different stimulants, simulating Gram-positive bacteria, a Gram-negative bacteria or a virus. Live Group A streptococci *S. pyogenes* serotype T1M1 obtained from the National Public Health Institute, Helsinki, Finland, grown as previously described^[19], was used as a Gram-positive bacteria at 1:1 host-cell:bacteria ratio; lipopolysaccharide (LPS) from *E. coli* serotype 0111:B4 (L-3024, Sigma) was used as a model for Gram-negative bacteria at a concentration of 100 ng/mL; and Influenza A H3N2 virus (A/Beijing/353/89) was used to infect cells at a multiplicity of infection of 5. Cell culture supernatants were collected individually at the 24 h time point and stored at -20°C before analysis.

Cytokine levels from cell culture supernatants of stimulated PBMC: Cytokine levels (TNF-α, IFN-γ, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10 and IL-12p70) in cell culture supernatants from each time point (baseline, 1 d, 7 d, 21 d and 3 wk after intervention) were determined using the FlowCytomix human Th1/Th2 10 plex kit II (BMS716FFCE) from Bender MedSystems (Vienna, Austria) according to manufacturer's instructions. The detection limit was 4.5 pg/mL for IL-1β, 8.9 pg/mL for IL-2, 6.4 pg/mL for IL-4, 5.3 pg/mL for IL-5, 4.7 pg/mL for IL-6, 6.4 pg/mL for IL-8, 6.9 pg/mL for IL-10, 7.9 pg/mL for TNF-α, 9.7 pg/mL for IL-12p70 and 7.0 pg/mL for IFN-γ. Only those cytokines from which over 80% of the samples were above the detection limit were statistically analyzed. Therefore, all unstimulated samples, IL-4 and IL-5 in all stimulated samples, and IFN-γ in LPS stimulated samples were not included in further analyses. For statistical analyses, samples under the detection limit were replaced by the values obtained by dividing the detection limit by two.

Saliva samples and secretory IgA

An unstimulated saliva sample was taken at every visit (at baseline, d 1, 7 and 21 and 3 wk after the intervention)

after the blood sampling. The saliva samples were placed in Eppendorf tubes, chilled, and stored at -20°C until secretory IgA was analyzed. SIgA from saliva was determined with an ELISA assay (catalog number K8870) purchased from Gentaur (Brussels, Belgium) according to the manufacturers' instructions.

Fecal samples and microbiological analyses

The fecal samples were collected at home at baseline and at the end of the 3-wk intervention period. Immediately after the collection the subjects were asked to deep-freeze (-20°C) the samples at home. They were subsequently transported to the study center on the morning of the study day and the samples were immediately put on dry ice and stored at -70°C until analysis. The amounts of the probiotic strains *L. rhamnosus* GG, *B. animalis* ssp. *lactis* Bb12 and *P. freudenreichii* ssp. *shermanii* JS in the fecal samples were analyzed with a previously described real-time quantitative PCR method^[20].

Study diary

Subjects were asked to fill in a structured study diary throughout the study. The study diary included questions about the use of the study product, the presence of any symptoms of respiratory infection, gastrointestinal symptoms or any other symptoms, the amount of exercise, and the use of any medication. No respiratory tract infections or major symptoms were recorded by the subjects during the study. The amount of weekly exercise carried out by the study subjects remained the same throughout the study.

Outcome measures and statistical analysis

The intention-to-treat population (all randomized patients who took at least one dose of the study product) was included in the analysis. The last-observation-carried-forward (LOCF) approach was used for missing data and for subjects who withdrew early.

The main outcome measures were the serum hsCRP levels and the cytokines produced by PBMCs. The responses for these outcomes were calculated as the area under the curve from the 0, 1 d, 7 d and 21 d, subtracted by the baseline value (AUC₀₋₂₁ minus baseline).

Data is presented as mean with standard deviation (SD) or as median with interquartile range (IQR). The differences between the groups were tested using the Kruskal-Wallis test or median regression analysis with Holm's adjustment for pair wise comparisons. A *P*-value below 0.05 was regarded as statistically significant, but no adjustment was made for multiple testing.

RESULTS

Highly sensitive CRP

In order to study the effect of probiotic bacteria on inflammatory markers, we determined serum CRP levels at different time points during the intervention. The median AUC₀₋₂₁ minus baseline (IQR) for hsCRP was 0.018 (-0.209-0.244) mg/L in the placebo group, -0.240 (-0.424-0.017) mg/L in the LGG group, 0.090 (-0.199-0.191) mg/L in the Bb12 group and -0.085 (-0.303-0.032) mg/L

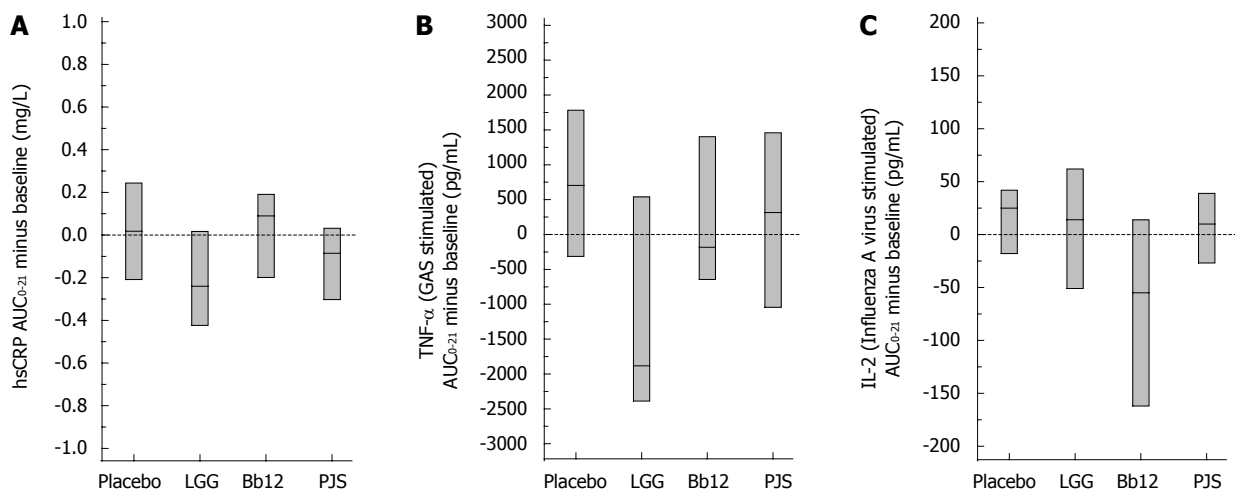


Figure 1 The median AUC₀₋₂₁ (minus baseline) with IQR for serum highly sensitive CRP (hsCRP) levels (A), for *Streptococcus pyogenes* (GAS)-stimulated TNF- α production from peripheral blood mononuclear cells (B) and for Influenza A virus-stimulated IL-2 production from peripheral blood mononuclear cells (C) during the 3-wk intervention period in healthy adults ($n = 62$). LGG: *Lactobacillus rhamnosus* GG; Bb12: *Bifidobacterium animalis* ssp. lactis Bb12; PJS: *Propionibacterium freudenreichii* ssp. *shermanii* JS.

Table 1 Counts of cells of innate and adaptive immunity ($10^9/L$) and levels of immunoglobulins (g/L) in serum and secretory IgA (g/mL) in saliva in healthy adults ($n = 62$) at baseline presented as median (IQR)

	Placebo ($n = 16$)	LGG ($n = 13$)	Bb12 ($n = 16$)	PJS ($n = 17$)	P value ¹
Leukocytes	4.90 (3.90-7.05)	5.20 (4.90-6.40)	5.25 (4.60-6.00)	4.90 (4.35-5.70)	0.55
Monocytes	5.00 (4.25-6.75)	5.00 (5.00-6.00)	6.00 (5.00-6.00)	6.00 (4.50-6.50)	0.84
Neutrophils	2.05 (1.67-3.72)	3.10 (2.50-3.30)	2.95 (2.12-3.47)	3.10 (2.15-3.45)	0.39
Basophils	0.05 (0.00-0.10)	0.10 (0.00-0.10)	0.05 (0.00-0.10)	0.00 (0.00-0.10)	0.73
Eosinophils	4.00 (3.00-5.75)	3.00 (2.00-6.00)	3.00 (2.25-4.75)	2.00 (1.50-3.00)	0.077
Lymphocytes	38.0 (33.5-48.7)	35.0 (31.5-37.5)	34.0 (30.0-40.5)	31.0 (26.0-40.5)	0.24
IgM	1.28 (0.97-1.65)	0.87 (0.69-1.32)	1.10 (0.73-1.66)	1.44 (0.80-1.77)	0.31
IgG	10.7 (9.5-12.2)	10.3 (9.3-11.7)	10.6 (9.1-12.1)	10.4 (8.4-11.7)	0.72
IgA	2.65 (2.45-3.21)	2.42 (2.04-3.44)	2.20 (1.73-2.94)	2.44 (1.53-2.85)	0.22
sIgA	0.23 (0.15-0.34)	0.27 (0.14-0.42)	0.40 (0.27-0.88)	0.28 (0.17-0.49)	0.065

¹Kruskal-Wallis test with Monte Carlo P values. IQR: Interquartile range.

in the PJS group ($P = 0.014$); a statistically significant difference was observed between LGG and Bb12 group by pair wise comparisons. In the LGG and PJS groups, hsCRP appeared to be at a lower level during the 3-wk intervention period compared with the Bb12 and placebo groups (Figure 1A).

Serum cytokines

The baseline values for pro-inflammatory cytokine TNF- α in serum were 1.2 pg/mL in the placebo group, 1.0 pg/mL in the LGG, 1.0 pg/mL in the Bb12 and 0.8 pg/mL in the PJS. The change (median with IQR) from baseline to the end of 3-wk intervention for TNF- α in these study groups was 0.1 (-0.1-0.3) pg/mL, 0.1 (-0.02-0.2) pg/mL, 0.3 (-0.04-0.4) pg/mL and 0.0 (-0.1-0.3) pg/mL, respectively ($P = 0.44$).

The baseline values for pro-inflammatory cytokine IL-6 were 0.3 pg/mL in the placebo group, 0.6 pg/mL in the LGG, 0.3 pg/mL in the Bb12 and 0.4 pg/mL in the PJS. The change (median with IQR) from baseline to the end of 3-wk intervention for IL-6 in these study groups was -0.5 (-0.6-0.0) pg/mL, -0.2 (-0.3-0.2) pg/mL, 0.1 (-0.3-0.3)

pg/mL and -0.04 (-0.3-0.1) pg/mL, respectively ($P = 0.26$). There were no statistically significant differences between the study groups with respect to serum cytokine levels.

Blood cells and immunoglobulins

Baseline values for leukocytes, monocytes, neutrophils, basophils, lymphocytes and immunoglobulins are presented in Table 1. There were no differences in these variables between the groups during the intervention.

Cytokines produced by PBMC

We also determined whether the use of probiotic bacteria has an effect on the overall responsiveness of PBMC to various microbial stimuli in *in vitro* cultured cells. The microbe-induced cytokine production by PBMC is presented in Table 2. *S. pyogenes*-stimulated production of pro-inflammatory cytokine TNF- α was significantly different between the groups ($P = 0.025$); a statistically significant difference was observed between LGG and placebo groups by pair wise comparisons (Figure 1B). Influenza A virus-stimulated production of Th1 cytokine IL-2 was significantly different between the groups ($P <$

Table 2 The effect of a 3-wk probiotic intervention on *in vitro* cytokine production (pg/mL) in peripheral blood mononuclear cells stimulated with *Streptococcus pyogenes*, lipopolysaccharide (LPS) from *E. coli* and Influenza A H3N2 virus of healthy adults (*n* = 62) presented as median AUC₀₋₂₁ minus baseline (IQR)

	Placebo (<i>n</i> = 16)	LGG (<i>n</i> = 13)	Bb12 (<i>n</i> = 16)	PJS (<i>n</i> = 17)	<i>P</i> value ¹	Localization
TNF- α						
<i>Streptococcus</i>	703 (-315-1784)	-1883 (-2389-540)	-645 (-1843-1403)	315 (-1045-1460)	0.025	LGG <i>vs</i> placebo
Influenza	31 (-3-66)	6 (-83-84)	-27 (-101-37)	29 (-39-83)	0.32	
LPS	11 (-16-38)	-15 (-31-10)	14 (-36-48)	-6 (-78-36)	0.53	
IFN- γ						
<i>Streptococcus</i>	-19 (-221-97)	-10 (-227-284)	71 (-161-360)	-23 (-223-155)	0.6	
Influenza	117 (29-284)	-72 (-221-194)	-7 (-436-189)	102 (-59-218)	0.25	
LPS	NA	NA	NA	NA		
IL-1 β						
<i>Streptococcus</i>	2308 (45-5222)	-1324 (-5609-352)	444 (-6152-5000)	649 (-3412-3747)	0.49	
Influenza	166 (13-478)	83 (-183-273)	-15 (-817-170)	156 (-75-791)	0.69	
LPS	67 (-29-138)	5 (-147-114)	51 (-40-122)	17 (-43-199)	0.66	
IL-2						
<i>Streptococcus</i>	5 (-23-79)	-46 (-176-0)	1 (-135-97)	39 (-105-213)	0.33	Bb12 <i>vs</i> others
Influenza	25 (-18-42)	14 (-51-62)	-55 (-162-14)	10 (-27-39)	< 0.001	
LPS	2 (-18-35)	27 (-25-57)	1 (-42-51)	6 (-35-25)	0.54	
IL-6						
<i>Streptococcus</i>	793 (-691-4829)	-379 (-3955-397)	205 (-2757-1553)	175 (-4344-909)	0.82	
Influenza	1530 (166-4632)	1288 (-4329-4073)	550 (-7178-1905)	1644 (-327-3344)	0.74	
LPS	947 (-418-2527)	-2189 (-3675-4053)	329 (-1949-3950)	639 (-1217-1607)	0.13	
IL-8						
<i>Streptococcus</i>	240 (-1585-3914)	34 (-3066-3143)	400 (-3638-2891)	132 (-2667-2976)	0.96	
Influenza	-455 (-1966-1670)	-193 (-2953-1520)	-1675 (-4245 to -601)	-1148 (-2550-1707)	0.31	
LPS	-742 (-3384-180)	-149 (-1608-1517)	-334 (-2692-739)	-1111 (-2457-836)	0.78	
IL-10						
<i>Streptococcus</i>	907 (263-2149)	-4 (-881-2420)	452 (-2982-1735)	226 (-86-950)	0.29	
Influenza	95 (48-284)	-57 (-159-233)	4 (-301-130)	95 (13-350)	0.25	
LPS	381 (19-602)	78 (-298-656)	347 (-403-796)	187 (28-1440)	0.51	
IL-12						
<i>Streptococcus</i>	26 (-18-75)	-32 (-99-36)	22 (-36-67)	35 (-73-172)	0.46	
Influenza	0 (-4-16)	7 (-9-30)	8 (-19-51)	5 (-25-37)	0.88	
LPS	15 (1-36)	3 (-7-28)	0 (-6-29)	0 (-62-20)	0.23	

¹Median regression analysis. AUC: Area under curve (calculated from baseline, 1, 7 and 21 d minus baseline); IQR: Interquartile range; LGG: *Lactobacillus rhamnosus* GG; Bb12: *Bifidobacterium animalis* ssp. *lactis* Bb12; PJS: *Propionibacterium freudenreichii* ssp. *shermanii* JS; NA: Not analyzed.

0.001); the statistically significant difference was observed between Bb12 and other groups (Figure 1C). There were no significant differences between the study groups with respect to the other cytokines produced by PBMC.

Detection of probiotic strains from feces

In order to determine whether the ingested bacteria could also be found in the fecal samples, the bacterial DNA levels were determined at the baseline and after the 3-wk intervention. The baseline levels for all three studied probiotics were low in fecal samples (Table 3). Despite the 3-wk run-in with probiotic restriction, a detectable level of the probiotic strains, especially LGG, was harbored in some of the subjects at baseline before the probiotic ingestion (Table 3). The amount of studied probiotic in feces in a given probiotic intervention group increased significantly from the baseline values during the intervention ($P < 0.001$). In the placebo group, the levels of different probiotics in feces remained low during the whole intervention period.

Follow-up samples

Three weeks after the intervention period, follow-up samples were taken. The levels for blood cells, immunoglobulins,

hsCRP and cytokines produced by PBMC were at the baseline levels.

DISCUSSION

In the present study, we studied the *in vivo* effects of three probiotic bacteria from three different genera on immune variables in healthy adults in a randomized, double-blind, placebo-controlled setting. The selection of these probiotics was based on our previous findings showing that, in human leukocyte cell cultures, probiotic bacteria readily induce cytokine production in PBMCs, but different bacteria are able to direct immune responses to either the Th1 type or the anti-inflammatory side in a genera-specific manner^[17]. Based on the cell culture results, two potentially anti-inflammatory strains (a *Bifidobacterium* and a *Propionibacterium* strain) and a well-studied *L. rhamnosus* GG strain^[21] as a reference probiotic were selected. Our data indicates that *in vivo* probiotics differ in their ability to induce anti-inflammatory and cytokine responses and may have a weak, genera-specific anti-inflammatory effect reflected as a decrease in serum hsCRP levels in healthy adults. In addition, we observed

Table 3 Detection of individual probiotic genomic DNA from fecal samples by quantitative PCR at baseline and after the 3-wk probiotic intervention in healthy adults ($n = 62$)

Strain	Fecal samples							
	Placebo ($n = 16$)		LGG ($n = 13$)		Bb12 ($n = 16$)		PJS ($n = 17$)	
	Baseline	After intervention	Baseline	After intervention	Baseline	After intervention	Baseline	After intervention
<i>L. rhamnosus</i> GG								
Number of subjects ¹	7	10	7	13	10	5	6	9
Mean (SD) ²	4.7 (1.2)	5.1 (1.1)	5.1 (1.4)	8.6 (0.6)	5.2 (1.3)	4.6 (1.5)	4.5 (1.2)	5.0 (1.4)
<i>B. animalis</i> ssp. <i>lactis</i> Bb12								
Number of subjects ¹	6	5	5	2	7	16	2	4
Mean (SD) ²	5.4 (1.6)	5.3 (1.7)	5.3 (1.4)	4.9 (1.3)	5.4 (1.6)	8.6 (0.5)	4.7 (1.1)	5.1 (1.6)
<i>P. freudenreichii</i> ssp. <i>shermanii</i> JS								
Number of subjects ¹	4	2	2	2	4	4	1	16
Mean (SD) ²	4.5 (1.4)	4.0 (0.7)	4.1 (0.8)	4.0 (0.6)	4.4 (1.6)	4.2 (0.9)	3.8 (0.3)	8.3 (1.0)

¹Number of subjects harboring a detectable level of the strain. ²Mean (\log_{10}) genome copies/g (SD). Detection limits for LGG and PJS is $3.7 \log_{10}$ genome copies/g and for Bb12 $4.3 \log_{10}$ genome copies/g.

that, during the intervention, *S. pyogenes*-induced TNF- α responses and influenza A virus-induced IL-2 responses in *in vitro* cultured PBMC were reduced, indicating a clear anti-inflammatory potential of some probiotic bacteria.

To our knowledge, this is the first study to show that probiotics may reduce serum hsCRP levels in healthy adults in a randomized, double-blind, placebo-controlled setting. It appeared that in the *L. rhamnosus* GG and *P. freudenreichii* ssp. *shermanii* JS treated groups, the hsCRP level tended to be lower during the intervention, whereas in *B. animalis* ssp. *lactis* Bb12 and the placebo groups, serum hsCRP levels remained unchanged. CRP is a sensitive marker of inflammation^[22] and provides an easy way to measure the anti-inflammatory potential of probiotics and other biological or pharmacological substances. This result was somewhat contradictory to our previous findings in leukocyte cell culture^[17], where *B. animalis* ssp. *lactis* Bb12 and *P. freudenreichii* ssp. *shermanii* JS were both good inducers of anti-inflammatory cytokines, whereas *L. rhamnosus* GG was a rather poor inducer of any cytokine. Previously, the effect of probiotics on CRP has only been studied in immunocompromised patients^[23-27], allergic children^[28] and patients suffering from rheumatoid arthritis^[29]. In immunocompromised patients, a combination of *L. casei*, *B. breve* and prebiotic galactooligosaccharides^[26] and *B. longum*^[30] have reduced serum CRP levels and also resulted in improvement in the overall clinical appearance of chronic inflammation^[30]. In contrast to the studies above and to our results in the present study, *Lactobacillus rhamnosus* GG increased serum hsCRP levels compared to placebo in infants with IgE-associated atopic eczema dermatitis syndrome^[28]. However, *L. rhamnosus* GG had no effect on serum CRP levels in patients with rheumatoid arthritis^[29]. It is of interest that a combination of four probiotic bacteria (*L. rhamnosus* GG, *L. rhamnosus* Lc705, *B. breve* 99, *P. freudenreichii* ssp. *shermanii* JS) did not have an effect on sensitive CRP^[28] in the same clinical setting with allergic children. In immunocompromised patients undergoing surgical procedures, *L. plantarum* 299V^[23,25] or a combination of *L. acidophilus* La5, *B. animalis* ssp. *lactis* Bb12, *S. thermophilus* and *L. bulgaricus*^[24,27] did not change serum CRP concentrations, either. It appears that the

effect of probiotics on CRP is controversial, and it is very difficult to compare the effects due to the differences in the measurement technique (highly sensitive *vs* normal CRP measurement), the different patient materials (healthy *vs* various diseases) and the different probiotic strains that have been used. It seems that age, the immunological status of the individual and the probiotic strain used in the study has a great impact on the immunomodulatory effects. Probiotics may have a strain-specific ability to lower serum CRP levels, thus having anti-inflammatory effects in apparently healthy adults and in patients suffering from different inflammatory conditions. In allergic patients, however, probiotics seem to induce a low-grade inflammatory response, as evidenced by increased serum CRP levels, and thus the treatment may have a beneficial effect on the host Th1/Th2 balance.

We found that *L. rhamnosus* GG was also able to reduce pro-inflammatory TNF- α production in the Gram-positive bacteria-stimulated PBMC. TNF- α is secreted by the monocytes, and it acts as an inflammatory mediator activating many types of cells. In our previous work with leukocyte cell culture, *L. rhamnosus* GG was found to be a relatively poor inducer of TNF- α , IL-12, IFN- γ and IL-10^[17]. Our present findings are supported by another clinical study carried out in healthy adults showing that *L. rhamnosus* GG treatment leads to decreased TNF- α production in PBMC^[31]. In addition, when the cytokine expression pattern in the small bowel mucosa was studied, it was found that *L. rhamnosus* GG induced the expression of genes involved in immune response and inflammation (TGF-beta and TNF family members, cytokines, nitric oxide synthase 1, defensin alpha 1)^[32]. Schultz and coworkers^[31] observed a decreased IL-6 and IFN- γ and an increased IL-10 and IL-4 production in PBMC obtained from *L. rhamnosus* GG treated individuals. We, however, did not find any significant changes in bacteria-induced production of cytokines apart from the TNF- α in the PBMC cultures of our study subjects after *L. rhamnosus* GG treatment. In another study with healthy adults and with patients with Crohn's disease, *L. rhamnosus* GG decreased the production of IL-2, IL-10 and IL-4 from PBMCs sorted as naïve and memory T cells^[33]. It seems that *L. rhamnosus* GG has a role in modulating the cytokine responses and may possess an

anti-inflammatory potential in healthy individuals.

In the present study, we also found that *B. animalis* ssp. *lactis* Bb-12 decreased the T lymphocyte growth factor IL-2 in the influenza-virus-stimulated PBMC, indicating an anti-inflammatory effect, which is consistent with our previous findings in human leukocyte cell culture^[17]. Our finding is a new one since, in healthy adults, a combination of *B. animalis* ssp. *lactis* Bb-12 and *L. paracasei* ssp. *paracasei* CRL-431 had no effect in *in vitro*-stimulated blood cytokine production^[7]. IL-2 is a very important cytokine in viral infections and inflammatory responses since it activates NK cells and induces activation and proliferation of T lymphocytes. Therefore, IL-2 production might be an important factor for a probiotic fighting against respiratory tract infections. Based on our present results, the *Bifidobacterium* strain might not be the most optimal strain against respiratory infections. Indeed, it is mainly probiotic strains from *Lactobacillus* genera-*L. rhamnosus* GG^[34], *L. casei* DN-114001^[35], a combination of *L. gasseri* PA 16/8, *B. longum* SP 07/3 and *B. bifidum* MF 20/5^[5-6], and *L. reuteri*^[36]-that have reduced the incidence or symptoms of common cold or respiratory tract infections. However, the immunomodulatory effects underlying the results observed in these studies have not been fully elucidated.

In conclusion, it appears that probiotics have an anti-inflammatory potential seen as a decrease in serum CRP levels and as a reduction in bacteria-induced production of pro-inflammatory cytokines in PBMC in healthy adults. However, all of the markers were in the normal range, and therefore the real impact of probiotics as anti-inflammatory substances warrants further evaluation in studies during inflammatory processes and with individuals suffering from various types of inflammatory or autoimmune diseases.

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COMMENTS

Background

Probiotics have been mostly studied in the prevention and treatment of different gastrointestinal diseases and allergy. Probiotic products, however, are usually consumed by the general, healthy population but not much is known what kind of effects they have on immune system in healthy adults.

Research frontiers

It is not fully clarified how probiotics exert their health effects, but one of the most probable action mechanisms is the modulation of immune responses *via* gut mucosal immune system.

Innovations and breakthroughs

In the present study the immunomodulatory effects of probiotics were studied in healthy adults. Probiotic bacteria had strain-specific anti-inflammatory effects reflected in reduced sensitive C-reactive protein, which is a new finding, and decreased proinflammatory cytokine production in peripheral blood mononuclear cells (PBMC).

Applications

Understanding of the specific immunomodulatory effects of probiotics may help in

designing future probiotics for targeted purposes. As the effects in the present study were investigated in healthy adults, the real impact of probiotics on inflammatory variables warrants further evaluation during inflammatory processes and with individuals suffering from various types of inflammatory or autoimmune diseases.

Peer review

The paper by Kekkonen and co-workers investigated the effects of three probiotic bacteria on immune variables in healthy adults. They observed strain-specific anti-inflammatory effects for distinct bacteria. Overall this paper is interesting and it has clearly stated aims, the sample size and the overall designs of the study are fair, the results adequate to provide experimental evidence and to support valid conclusions. As placebo per se could cause effects on immune response, a further control group, formed by healthy subjects, would be advisable in order to analyze the basic fluctuation of all the parameters studied.

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A combination therapy of ethanol injection and radiofrequency ablation under general anesthesia for the treatment of hepatocellular carcinoma

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energy could be applied during treatment under pain-free condition for the patients.

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Abstract

AIM: To summarize the effects of laparoscopic ethanol injection and radiofrequency ablation (L-EI-RFA), thoracoscopic (T-EI-RFA) and open-surgery assisted EI-RFA (O-EI-RFA) under general anesthesia for the treatment of hepatocellular carcinoma (HCC).

METHODS: Time-lag performance of RFA after ethanol injection (Time-lag PEI-RFA) was performed in all cases. The volume of coagulated necrosis and the applied energy for total and per unit volume coagulated necrosis were examined in the groups treated under general (group G) or local anesthesia (group L).

RESULTS: The results showed that the total applied energy and the applied energy per unit volume of whole and marginal, coagulated necrosis were significantly larger in group G than those in the group L, resulting in a larger volume of coagulated necrosis in the group G. The rate of local tumor recurrence within one year was extremely low in group G.

CONCLUSION: These results suggest that EI-RFA, under general anesthesia, may be effective for the treatment of HCC because a larger quantity of ethanol and

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most serious malignancies worldwide^[1], especially in Asian countries due to the high exposure to hepatitis virus. Despite intensive efforts to develop novel treatment modalities for HCC, the prognosis of HCC remains relatively poor^[2-4]. Although percutaneous ethanol injection (PEI) and percutaneous acetate injection (PAI) are frequently used for the treatment of HCC, these treatment modalities are considered to be effective for patients with relatively small, encapsulated HCC. By contrast, tumor ablation technologies such as microwave, laser, and radiofrequency ablation (RFA), have been shown to be reliable and effective for inducing thermally mediated coagulation necrosis of primary HCC^[5-10] and metastatic liver cancer^[11,12]. Among these treatment modalities, it is now possible to obtain larger areas of coagulated necrosis through innovative RFA technologies, while RFA now plays a central role in local treatment due to wider coagulated necrosis in fewer sessions without major complications compared with PEI and PAI. However, in contrast to its efficacy, several disadvantages have been pointed out, such as the limited

coagulated necrosis induced by RFA and frequent local tumor recurrences^[13,14]. To overcome these weaknesses, we have developed a novel combination therapy of percutaneous ethanol injection and radiofrequency ablation (PEI-RFA) and showed that combined use of ethanol prior to RFA was able to enhance the therapeutic effects with a smaller energy requirement compared with RFA alone^[15-20]. Although PEI-RFA was shown to enlarge the area of coagulated necrosis, there are HCC cases that are difficult to treat with percutaneous RFA due to the location of the HCC. For example, HCC protruding from the surface of the liver are difficult to treat with RFA percutaneously due to the risk of tumor bleeding. Furthermore, HCC located closely to the diaphragm are difficult to treat with RFA percutaneously due to the poor visualization of the tumor by ultrasonography (US). For these HCC thus other appropriate RFA approaches are desirable. Furthermore, there are some HCC that can not be treated even with both laparoscopic and thoracoscopic approaches due to the location of the tumor, and open surgery-assisted RFA remains the only appropriate treatment. For these kinds of HCC, we applied the combination therapy of ethanol injection and RFA (EI-RFA) to laparoscopic, thoracoscopic, and open-surgery assisted treatments. We summarized and evaluated the effectiveness of laparoscopic-EI-RFA (L-EI-RFA), thoracoscopic-EI-RFA (T-EI-RFA), and open surgery-assisted-EI-RFA (O-EI-RFA) under general anesthesia.

MATERIALS AND METHODS

Patients

L-EI-RFA was performed in eight patients (5 male and 3 female; mean age 67 years) with HCC protruding from the surface of the left lobe of the liver (ranging from 1.0-3.0 cm in diameter). T-EI-RFA was performed in nine patients (6 male and 3 female; mean age 69 years) with HCC located closely to the diaphragm (ranging from 1.0-3.5 cm in diameter). O-EI-RFA was performed in five patients (4 male and 1 female; mean age 63 years) with HCC located closely to the diaphragm (ranging from 1.0-2.5 cm in diameter). P-EI-RFA was performed in 40 patients (27 male and 13 female; mean age 65 years). All these studies were conducted with informed consent at the time of enrollment. The characteristics of the enrolled patients are shown in Table 1.

Treatment of time-lag EI-RFA

We previously reported that time-lag performance RFA after ethanol injection (time-lag P-EI-RFA) was effective for the treatment of HCC^[20]. Time-lag PEI-RFA was performed under the real-time US guidance with a 3.5-MHz sector probe (TOSHIBA, Xario Prime Ultrasound, SSA-660A). RFA was performed by a Cool-tip RF System (RADIONICS, Burlington, USA)^[21], a RTC system (RF3000 Generator, Boston Scientific, USA), and RITA system (Model 90, USA) according to the method described in our manuscripts^[15,16]. Briefly, a 17-gauge RFA needle with an electrode of 3 cm in length was inserted into the center of tumor, followed by a 21-gauge PEI needle inserted into the liver tumor through the same

Table 1 Characteristics of patients enrolled in the present study

	Group G	Group L
Number of patients	22	40
Male/Female	15/7	27/13
Age (yr)		
Mean	64	65
Range	48-75	49-72
Tumor size (cm)		
Mean	2.8	2.9
Range	1.0-3.5	1.5- 3.3
Injected ethanol (mL)		
Mean	5.2	2.1
Range	2.0-15	1.5-2.5
Child-Pugh grade		
A	14	25
B	7	14
C	1	1

attachment hole beside the echo probe. Pure ethanol (99.8%) was then slowly injected into the tumor. The volume of injected ethanol was always kept below double the estimated tumor volume. Five minutes after injection of ethanol into the tumor, the ablation was started with 30 W of power output followed by a stepwise increase of 20 W every 2-3 min. After the end of the ablation (50 W of power output), the circulating cooling water was stopped and the temperature of the RFA electrode was checked. The ablation was performed under impedance control. The ablation was terminated when the temperature of the RFA needle was > 65°C.

Laparoscopic time-lag EI-RFA (L-EI-RFA)

L-EI-RFA was performed for the HCC protruding from the surface of the liver. First, the laparoscope was inserted into the abdominal cavity beginning in the left-upper portion of the navel. RFA electrode and PEI needle were inserted at a second abdominal site according to the location of the tumor. A sonde was inserted into the abdominal cavity to lift up the liver when the HCC was protruding from the reverse surface of the liver. Under laparoscopic observation, a RFA electrode was directly inserted into the tumor, and then a 21-gauge PEI needle was inserted and ethanol was injected^[18].

Thoracoscopic time-lag EI-RFA (T-EI-RFA)

T-EI-RFA was performed for HCC close to the right diaphragm. Patients were put under general anesthesia with one-lung (left lung) ventilation in a left-decubitus position. Three ports for the thoracoscope, End-fire laparoscopic probe (ALOKA UST-52109), and water-pouring tool were inserted into the pleural cavity through the intercostal space. After putting the collapsed right lung aside by a laparoscope, the End-fire laparoscopic probe was guided to the surface of the exposed diaphragm, and the tumor close to the diaphragm was identified by US (ALOKA ProSound SSD-3500). After visualizing the tumor by US, an RFA electrode was inserted into the tumor through the channel along the End-fire laparoscopic probe. A 21-gauge

Table 2 Comparison of the volume of coagulated necrosis and energy requirement between the groups treated with expandable and straight electrode

	T-S (cm)	ETOH (mL)	L (cm)	S (cm)	H (cm)	V (cm ³)	M (cm ³)	T-ENE (J)	T-ENE/V (J/cm ³)	T-ENE/M (J/cm ³)
Group L (n = 40)	2.0 ± 0.8	3.0 ± 1.9	3.5 ± 0.6	2.8 ± 0.4	3.0 ± 0.5	15.9 ± 5.2	10.1 ± 8.2	21565 ± 9631	1636 ± 791	2406 ± 1819
Group G (n = 15)	2.1 ± 0.8	6.1 ± 3.4	3.6 ± 1.1	3.2 ± 0.6	3.2 ± 0.8	22.5 ± 14.9	15.9 ± 12.1	37207 ± 20583	2409 ± 2045	3594 ± 2727
P value	0.56	0.0007	0.64	0.10	0.31	0.045	0.06	0.0095	0.10	0.11

Twenty-four HCC were treated with time-lag PEI-RFA with straight type electrode, while 15 HCC were treated with time-lag PEI-RFA with expandable type electrode. After treatment, the longest and the shortest diameters, and the height of the coagulated necrosis were estimated by helical dynamic CT and the approximation volume of total and marginal coagulated necrosis were calculated. Each datum expresses mean ± SD. Each abbreviation in the table is expressing as follows: T-S: Tumor size; ETOH: Amount of ethanol; L: Longest diameter; S: Shortest diameter; H: Height; V: Volume of coagulated necrosis; M: Volume of marginal coagulation; D: Duration of ablation; T-ENE: Total energy requirement; T-ENE/V: Energy requirement per unit volume for whole coagulation; T-ENE/M: Energy requirement per unit volume for inducing marginal coagulation.

PEI needle was inserted and pure ethanol or lipiodol containing ethanol was injected^[19].

Open surgery-assisted time-lag EI-RFA (O-EI-RFA)

O-EI-RFA was performed for HCC which were difficult to treat with other EI-RFA approaches or when splenectomy was simultaneously performed to improve cirrhotic liver dysfunction accompanying severe esophageal varices or a decrease of platelet count. After exposing the liver and confirming the surface location of liver tumors by US, the RFA electrode was directly inserted into the liver tumors and time-lag EI-RFA was performed.

Evaluation of therapeutic efficacy

Five to seven days after treatment, plain or contrast enhanced CT was performed to evaluate the response to L-EI-RFA, T-EI-RFA, and O-EI-RFA. Tumor necrosis was considered to be complete when no foci of early enhancement were seen around the original regions.

Statistical analysis

Statistical analysis was performed using Statview II (Version 5.0), statistical significance between the group L and group G was analyzed by a Chi-square test for independence and significant difference was accepted at $P < 0.05$.

RESULTS

Comparison of the volume of coagulated area and the applied energy requirement for total and unit volume coagulation in patients treated with EI-RFA under local anesthesia (group L) and general anesthesia (group G)

Group L (40 cases) received time-lag EI-RFA under local anesthesia, while group G (22 cases) received time-lag EI-RFA under general anesthesia. The patients underwent RFA therapy by means of the Cool-tip RF system, RTC system, and RITA system. No major adverse effects were observed in either group. Among treated cases, 40 cases in group L and 15 cases in group G (L-EI-RFA; 6 cases, T-EI-RFA; 8 cases, O-EI-RFA; 1 case) were treated with EI-RFA by means of the Cool-tip RF system. Between these patients treated by the Cool-tip RF system, the effect of EI-RFA was compared using several parameters drawn from the treatments. Comparison of the amount of injected ethanol, the volume of the induced coagulated necrosis, total applied energy for total and per unit volume coagulated necrosis

in both groups are summarized in Table 2. The tumor size was approximately 3 cm in diameter in both groups, and no significant difference was detected between groups. Although the longest and the shortest diameter and the height of the coagulated necrosis did not show a significant difference between the groups, the mean values of all of these parameters in group G were higher than those in group L. Thus, the volume of total coagulated necrosis in group G was significantly larger than that in group L. Furthermore, the volume of marginal coagulated necrosis in group G was also larger than that in group L. According to the analysis of parameters which affect the volume of induced coagulated necrosis in EI-RFA, both the quantity of ethanol and applied energy for ablation in group G were significantly larger than those in group L. Because both, the volume of coagulated necrosis and total applied, energy, were increased in group G compared with group L, the applied energy per unit volume for whole and marginal coagulated necrosis were comparable. These results suggest that a higher volume of coagulated necrosis was induced in group G compared with group L because higher amounts of ethanol and energy for ablation could be applied.

Rate of local recurrence within a year in group G and L

Among the 22 patients treated with EI-RFA under general anesthesia (group G), local recurrence was observed in only one case (4.5%) within a year after the treatment. By contrast, local recurrence was detected within a year in four cases (10%) among the 40 cases treated with EI-RFA under local anesthesia (group L). Although the difference of the rate of local recurrence between group G and group L did not reach statistical significance, the rate was extremely low in group G. In one case (patient No. 21) with local recurrence, the volume of marginal coagulated necrosis around the original tumor was lower than in cases without recurrence.

Comparison of the effects of L-EI-RFA, T-EI-RFA, and O-EI-RFA

The results for treatment with L-EI-RFA, T-EI-RFA, and O-EI-RFA are summarized in the Table 3. L-EI-RFA was performed on the HCC located in the left lobe of the liver (segment 2-4) and T-EI-RFA was performed on the HCC of segment 8 of the liver close to the diaphragm (except for one HCC located in the segment 6). O-EI-RFA was performed on the liver HCC of segment 3, 7, and 8. The

Table 3 Results of L-EI-RFA, T-EI-RFA and O-EI-RFA

Location		T-S (cm)	Ins	EtOH (mL)	A-A (L × S × H) (cm)	V (cm ³)	M (cm ³)
L-EI-RFA							
No. 1	S3	1.5	Cool-tip	15	3.2 × 2.7 × 2.6	11.8	10.0
No. 2	S2	3.0	Cool-tip	8	4.2 × 4.2 × 4.2	38.7	24.0
No. 3	S4	1.5	Cool-tip	2	2.2 × 2.2 × 2.2	5.60	3.81
No. 4	S2	3.0	Cool-tip	7	6.7 × 5.0 × 3.7	64.8	50.7
No. 5	S3	2.5	Cool-tip	8	3.7 × 3.7 × 3.4	24.4	16.2
No. 6	S4	1.0	Cool-tip	7	2.2 × 2.2 × 2.3	5.83	5.30
No. 7	S2	1.5	RITA	2	4.0 × 4.0 × 4.0	33.0	31.0
No. 8	S2	1.5	RITA	2	3.5 × 2.5 × 3.0	19.0	17.0
T-EI-RFA							
No. 9	S6	2.5	Cool-tip	7	3.7 × 2.2 × 3.2	19.8	11.7
No. 10	S8	1.0	RTC	3	4.1 × 2.5 × 3.2	17.2	16.6
No. 11	S8	2.0	Cool-tip	12	4.2 × 4.2 × 3.7	34.1	30.0
No. 12	S8	2.5	Cool-tip	5	3.2 × 3.2 × 3.2	17.1	8.97
No. 13	S8	2.0	Cool-tip	4	3.0 × 3.0 × 2.5	11.8	7.59
No. 14	S8	2.0	Cool-tip	5	3.5 × 3.5 × 3.5	22.4	18.3
No. 15	S8	2.0	Cool-tip	5	4.5 × 3.0 × 3.5	24.7	20.5
No. 16	S8	3.5	Cool-tip	3	2.0 × 2.0 × 2.0	4.20	3.70
No. 17	S8	2.0	Cool-tip	4	2.5 × 2.5 × 4.0	13.1	8.90
O-EI-RFA							
No. 18	S3	1.5	RITA	2	3.3 × 2.5 × 3.0	13.0	8.90
No. 19	S8	2.5	Cool-tip	7	4.2 × 3.2 × 3.7	26.0	17.9
No. 20	S7	1.0	RTC	2	3.0 × 2.5 × 2.5	9.81	9.29
No. 21	S3	1.5	RTC	2	2.0 × 2.0 × 1.5	3.14	1.37
No. 22	S3	1.5	RTC	2	4.0 × 4.0 × 4.0	25.1	23.4

Eight HCC were treated with L-EI-RFA, Nine HCC were treated with T-EI-RFA and five HCC were treated with O-EI-RFA. Location of the tumor, tumor size (T-S), instruments for ablation (Ins), amounts of injected ethanol (EtOH), ablated area (A-A), [longest diameter (L) × shortest diameter (S) × height (H)], volume of coagulated necrosis (V) and volume of marginal coagulated necrosis (M) are shown.

approximate estimated volume of the original tumor, volume of whole and the marginal coagulated necrosis were calculated from the CT image after the treatments. In most treated cases, a larger volume of coagulated necrosis and marginal coagulated necrosis was induced compared with the volume of original tumor. In most patients treated with a large amount of ethanol (over 7 mL), larger volume of whole and marginal coagulated necrosis were induced in patients such as No. 2, 4, 5, 9, 11, and 19. The volume of whole and marginal coagulated necrosis was comparable in the patients treated with L-EI-RFA, T-EI-RFA, and O-EI-RFA.

Relationship between the amount of ethanol, applied energy, and the volume of coagulated necrosis

In group G, the induced coagulated necrosis increased with the amount of ethanol and applied energy. We previously showed that the amount of ethanol was positively correlated with the volume of coagulated necrosis in patients treated with P-EI-RFA using an RFA instrument equipped with a straight electrode (Cool-tip RF system)^[19]. In the present study, the relationship between the amount of ethanol or applied energy and the volume of induced coagulated necrosis were evaluated in patients treated with EI-RFA under general anesthesia. Furthermore, the relationship between the product of the amount of ethanol and the applied energy vs the volume of coagulated necrosis was also analyzed. The results showed that both the amounts of injected ethanol and applied energy were significantly and positively correlated with the volume of

coagulated necrosis (Figure 1A and B). The product of the amount of ethanol and the applied energy was also positively correlated with the volume of coagulated necrosis (Figure 1D). These results clearly indicated that both the amount of ethanol and applied energy were critical factors that regulate the volume of coagulated necrosis in the EI-RFA under general anesthesia.

Representative case treated with L-EI-RFA

By analyzing the effects of time-lag EI-RFA under general anesthesia, one point became evident: Total applied energy and the applied energy per unit volume of whole and marginal coagulated necrosis were significantly larger in patients treated under general anesthesia than in those treated under local anesthesia. This led to the induction of a larger volume of coagulated necrosis. A representative case treated with T-EI-RFA was shown in our previous manuscript^[22]. A case treated with L-EI-RFA is presented. One case with HCC successfully treated with L-EI-RFA is shown in Figure 2. An HCC (2 cm in diameter) was located in the S2 region of the liver protruding from the reverse surface and with an enhanced early vascular phase (Figure 2A) *via* dynamic CT. The laparoscope was inserted from the left-upper portion of the abdomen into the abdominal cavity and the liver was lifted by a sonde. An RFA electrode and PEI needle were percutaneously inserted into the center of the tumor, monitoring the depth of the inserted electrode by the linear type US. The electrode and needle were firstly inserted into the tumor from the upper surface of the liver (Figure 2B), and then inserted into the

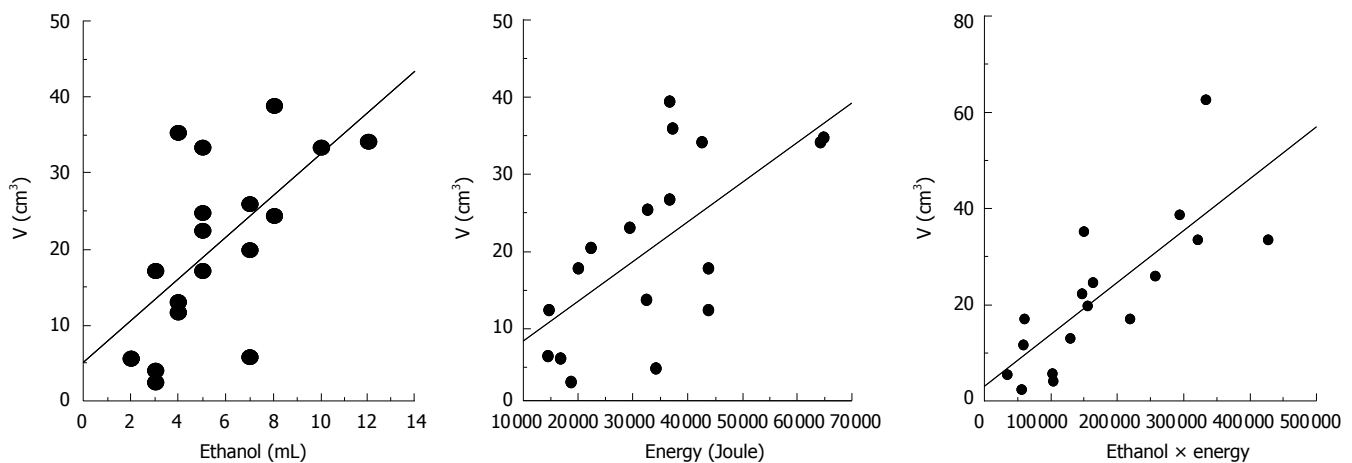


Figure 1 Relationship between the volume of coagulated necrosis induced and the amounts of ethanol injected or total applied energy or the product of the amounts of ethanol and the total applied energy. All the amounts of ethanol, total applied energy, or the products of the amount of ethanol and total applied energy showed a significant positive correlation with the volume of coagulated necrosis. (Ethanol vs volume, $r = 0.54$, $P = 0.018$; energy vs volume, $r = 0.61$, $P = 0.0057$; ethanol \times energy vs volume, $r = 0.61$, $P = 0.0078$).

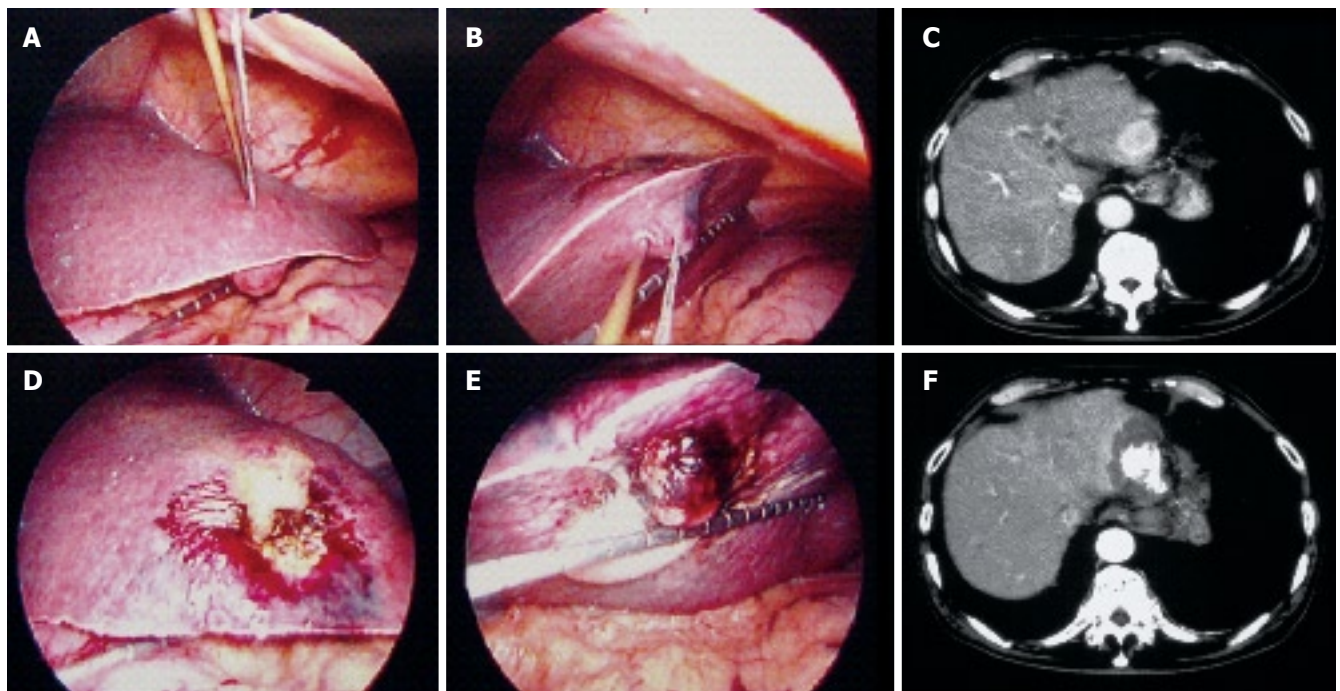


Figure 2 A case of HCC located on the reverse surface of the liver (S2 of the liver) treated with L-EI-RFA. A HCC (2 cm in diameter) showed an enhancement in early vascular phase of helical dynamic CT (A); RFA electrode and PEI needle were firstly inserted from the surface of the liver (B) and secondly inserted from the reverse surface of the liver (C); After injecting the ethanol containing 15% lipiodol, RFA was performed. Dynamic CT after the treatment showed lipiodol deposit associated with the original tumor and low density area was observed around the tumor (D); Laparoscopic observation of the tumor from the surface (E) and from the reverse surface (F) of the liver after the treatment.

tumor from the reverse surface of the liver (Figure 2C). We previously reported the usefulness of injecting the mixture of ethanol and lipiodol to visualize the original tumor by dynamic CT^[23]. Therefore, a mixture of ethanol and lipiodol (15% lipiodol in ethanol) was injected into the tumor. Five minutes after injection of ethanol containing lipiodol, RFA was started at 30 W, and the power output was stepwise increased to 80 W by a Cool-tip RF system. During ablation, the tumor was constantly lifted by a sonde to prevent the transmission of heat to the mesentery. Abdominal dynamic CT taken after the operation clearly

showed a lipiodol deposit associated with the tumor and the ablated region reached beyond the tumor (Figure 2D). A safety margin was shown to be sufficiently obtained by L-EI-RFA. The laparoscopic findings for the tumor after ablation are shown in Figure 2E and F.

DISCUSSION

HCC is one of the most serious and common malignancies worldwide^[1,4]. As a treatment for HCC, RFA now plays a central role for local control of HCC, because RFA

can induce wider coagulated necrosis in a few sessions compared with PEI which is frequently used for relatively small-encapsulated HCC. However, the region of coagulated necrosis induced by RFA is still limited and only considered applicable to tumors within a 3 cm diameter. Furthermore, it is also pointed out that relatively frequent local recurrences of tumor occur after RFA treatment. Therefore, the RFA technique could be further developed to improve the therapeutic effects of this treatment. To enhance the therapeutic effect of RFA, several treatment modalities have been applied in addition to local treatment^[24-29]. As one of the optional combination therapies, we have developed a novel combination therapy of P-EI-RFA and showed that this combination therapy accurately enlarged the area of induced coagulated necrosis. Total applied energy and the applied energy per unit volume of whole and marginal coagulated necrosis was significantly lower in the P-EI-RFA than RFA alone. Furthermore, we found that the time-lag performance of RFA after ethanol injection (time-lag P-EI-RFA) resulted in a lower energy requirement per total and unit volume of coagulated necrosis than without time-lag performance of RFA after ethanol injection. In this regard, we suggest that time-lag P-EI-RFA can induce wider coagulated necrosis with a smaller energy requirement. Although P-EI-RFA was shown to enlarge the area of coagulated necrosis, there are HCC cases that are difficult to treat with the percutaneous RFA due to the location of the HCC. For these situations, we applied the combined therapy of ethanol injection and RFA (EI-RFA) with laparoscopic, thoracoscopic and open-surgery assisted. Among 22 patients treated with EI-RFA under general, the number of local recurrences was very small [1 case (4.5%)] and its frequency was kept in extremely low level. Analysis of the amount of injected ethanol, applied energy and the volume of coagulated necrosis showed that these parameters in the group treated with EI-RFA under general anesthesia were significantly larger than those in the group treated under local anesthesia. One of the most relevant differences between the EI-RFA under general anesthesia and local anesthesia is presence or absence of pain felt by the treated patients. We have reported in a series of analyses that P-EI-RFA under local anesthesia enabled a comparable coagulated necrosis with smaller energy requirement relative to RFA alone. P-EI-RFA was likely to be less invasive than RFA alone^[16]. However, in the present study, the rate of local recurrence was reduced in the patients treated under general anesthesia compared with the patients treated under local anesthesia. Taken collectively, these results suggest that higher amounts of ethanol and energy administered under pain-free conditions may result in a decreased rate of local tumor recurrences after RFA treatment. Indeed, although we still believe that PEI-RFA is less invasive for the treatment of HCC, we are sometimes obliged to cease the RFA treatment due to the pain felt by the patient during the percutaneous RFA treatment. Therefore, the results in the present study suggest that it is important to apply enough ethanol and energy for RFA treatment to decrease the local recurrence after percutaneous treatment as well as treatment under general anesthesia. For this purpose, it is beneficial to use anesthesia intravenously to decrease the

pain felt by the patients during the percutaneous treatment as well. Patients under pain-free conditions during treatment may have a decreased rate of local tumor recurrence. Recently, it was reported that there were no differences in tumor control and complications under general anesthesia and analog-sedation in RFA treatment of pulmonary tumors^[30]. This result is not in accordance with our results obtained during treatment of HCC. In the treatment of HCC located near the surface of the liver, patients often complain about pain originating from the membrane of the liver. In our patients, we usually use pentazocine and non-steroid anti-inflammatory drugs (NSAIDs) (if necessary diazepam is also used on a case by case basis) for the percutaneous RFA treatment. Therefore, it may be better to consider stronger pain relief during the treatment of percutaneous RFA treatment.

In conclusion, we compared the clinical effects, amounts of ethanol, and applied energy in P-EI-RFA between patients under general anesthesia and local anesthesia. The volume of induced coagulated necrosis, amounts of ethanol, and applied energy were significantly larger in the group treated under general anesthesia than that under local anesthesia. The rate of local tumor recurrence in the former group was kept at an extremely low level.

COMMENTS

Background

Radiofrequency ablation (RFA) plays a central role for the treatment of hepatocellular carcinoma (HCC) because this newly developed technology appears very effective to induce wider coagulated necrosis. However, several disadvantages have been pointed out for RFA and improvement of RFA technique will be desirable.

Research frontiers

RFA treatments are performed percutaneously under local anesthesia in many cases. Local tumor recurrence varies according to the location of tumor in the liver, size of tumors, and level of RFA technique. A few reports compared the effects of RFA treatment under local and general anesthesia.

Innovations and breakthroughs

This report showed that the total applied energy and the applied energy per unit volume of whole and marginal coagulated necrosis were significantly larger in the group treated under general anesthesia (group G) resulting in a larger volume of coagulated necrosis.

Applications

Patients under pain-free condition during treatment may have a decreased rate of local tumor recurrence. It thus may be better to consider stronger pain relief during the treatment of percutaneous RFA treatment.

Peer review

Dr. Kurokohchi and colleagues reported the advantage and features of the combination therapy of ethanol injection and radiofrequency ablation (EI-RFA) under general anesthesia for HCC. This manuscript arouses interest for readers and provides an important clue to effectively treat patients with HCC.

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RAPID COMMUNICATION

Serum type IV collagen level is predictive for esophageal varices in patients with severe alcoholic disease

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detection of esophageal varices in SAD.

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Mamori S, Searashi Y, Matsushima M, Hashimoto K, Uetake S, Matsudaira H, Ito S, Nakajima H, Tajiri H. Serum type IV collagen level is predictive for esophageal varices in patients with severe alcoholic disease. *World J Gastroenterol* 2008; 14(13): 2044-2048 Available from: URL: <http://www.wjgnet.com/1007-9327/14/2044>. asp DOI: <http://dx.doi.org/10.3748/wjg.14.2044>

Abstract

AIM: To determine factors predictive for esophageal varices in severe alcoholic disease (SAD).

METHODS: Abdominal ultrasonography (US) was performed on 444 patients suffering from alcoholism. Forty-four patients found to have splenomegaly and/or withering of the right liver lobe were defined as those with SAD. SAD patients were examined by upper gastrointestinal (UGI) endoscopy for the presence of esophageal varices. The existence of esophageal varices was then related to clinical variables.

RESULTS: Twenty-five patients (56.8%) had esophageal varices. A univariate analysis revealed a significant difference in age and type IV collagen levels between patients with and without esophageal varices. A logistic regression analysis identified type IV collagen as the only independent variable predictive for esophageal varices ($P = 0.017$). The area under the curve (AUC) for type IV collagen as determined by the receiver operating characteristic (ROC) for predicting esophageal varices was 0.78.

CONCLUSION: This study suggests that the level of type IV collagen has a high diagnostic accuracy for the

INTRODUCTION

Regular daily drinking is more likely to result in liver damage than intermittent drinking. The longer this pattern is maintained, the more likely it is that alcoholic hepatitis, and subsequently cirrhosis, will develop^[1]. In patients with cirrhosis, the incidence of esophageal varices increases by nearly 5% per year, and the rate of progression from small to large varices is approximately 5%-10% per year^[2,3]. Bleeding from esophageal varices is common among patients with cirrhosis. Bleeding from varices may occur in 15%-68% of patients with varices^[4]. Variceal hemorrhaging is associated with a high mortality and with high hospital costs^[5]. Both, beta-blockers and endoscopic procedures, have been established as effective preventive modalities for variceal hemorrhage^[5,6]. Therefore, the early detection of esophageal varices is critical for the effective prevention of variceal hemorrhage^[7]. Adding an accurate serum marker for hepatic fibrosis to the model may improve the diagnostic accuracy in predicting esophageal varices without performing liver biopsy. Moreover, developing an accurate non-invasive diagnostic model might also decrease the costs for the prevention of hemorrhaging from varices^[7].

In daily medical practice, it is common to encounter

patients with liver damage from chronic alcohol consumption. Moreover, when the alcoholic patient is examined, it is often evident that alcoholic liver damage is progressing. Once alcoholic cirrhosis is established, esophageal varices develop in the majority of patients, as found during prolonged follow-up^[8]. Nevertheless, alcoholic patients tend to be indifferent regarding self health, and are not likely to undergo periodic consultations. We therefore examined predictive factors for esophageal varices in severe alcoholic disease.

MATERIALS AND METHODS

Patients

The 444 consecutive patients considered for this study were hospitalized at the Tokyo Medical Center of Alcohol Related Disabilities in Tokyo, Japan, between April and September 2005, July 2006, and June 2007 for alcoholism.

A complete physical examination was performed by a senior physician. The recorded variables included age, gender, height, body weight, mean alcohol consumption, duration of alcohol abuse, jaundice, ascites, and hepatic encephalopathy.

After an overnight fast, serum samples were obtained from all patients for test purposes, including a complete blood cell count, blood platelets, bilirubin, aspartate transaminase (AST), alanine aminotransferase (ALT), gamma glutamyl transpeptidase (GTP), albumin, prothrombin index (ratio between patient and control Quick time expressed in percentage), and type IV collagen (Mitsubishi Chemical Medience Corporation, Tokyo, Japan).

Ultrasonography (US) was performed by experienced gastroenterologists during the stay in hospital. The spleen was visualized with the patient in the right lateral decubitus position. Measurements were then taken in the sagittal (S) and transverse (T) planes, with the maximum dimension being recorded in each plane^[9]. Splenomegaly was defined as a spleen index ($S \times T \times 0.9$) > 30. Alcoholic patients with splenomegaly and/or withering of the right liver lobe were defined as severe alcoholic disease patients (SAD) and included into the study. Patients with the following criteria were excluded: (1) the presence of suspected hepatocellular carcinoma on US; (2) the presence of extra-hepatic infectious or inflammatory disease; (3) treatment by any drug known to affect liver fibrosis; (4) seropositivity for the hepatitis B surface antigen, hepatitis C virus, and/or human autoimmune antibodies. Finally, we identified 44 SAD patients (Table 1).

For each patient, upper gastrointestinal (UGI) endoscopy was performed by an endoscopist. The purpose of endoscopy was to evaluate the presence of esophageal varices (Figure 1). The endoscopist evaluated esophageal varices with the Esophagogastric Varices Grading System of the Japan Society for Portal Hypertension^[10]. The endoscopist performed UGI endoscopy without knowledge of serum data.

Statistical analysis

The results were expressed as the mean \pm SD. Differences between the groups were examined for statistical signifi-

Table 1 Characteristics of the study population ($n = 44$)

Esophageal varice	Yes ($n = 25$)	No ($n = 19$)	P value
Age (yr)	49.6 \pm 7.0	55.5 \pm 9.2	< 0.05
Sex (male/female)	20/5	15/4	NS
Total alcohol intake (kg)	1075.9 \pm 646.9	1018.4 \pm 684.9	NS
MCV (fL)	97.7 \pm 12.4	95.9 \pm 12.0	NS
Plt (/ μ L)	12.6 \pm 6.1	13.9 \pm 9.8	NS
PT (%)	62.6 \pm 16.2	69.3 \pm 18.8	NS
AST (IU/L)	80.9 \pm 72.6	96.1 \pm 85.1	NS
ALT (IU/L)	44.4 \pm 2.6	45.2 \pm 28.5	NS
GTP (IU/L)	453.6 \pm 594	410.2 \pm 374.3	NS
T-Bil (mg/dL)	2.7 \pm 3.2	2.4 \pm 1.9	NS
Alb (g/dL)	3.7 \pm 0.6	3.9 \pm 0.5	NS
Collagen type IV (ng/mL)	712.3 \pm 355.6	404.3 \pm 198	< 0.001
Ascites (yes/no)	6/19	2/17	NS
Encephalopathy (yes/no)	1/24	2/17	NS

NS: Not significant; Normal ranges: MCV (mean corpuscular volume), 85-102 fL; Plt (platelet count), $14-34 \times 10^3/\mu$ L; PT (prothrombin index), 70%-100%; AST (aspartate aminotransferase), 10-40 IU/L; ALT (alanine aminotransferase), 5-45 IU/L; GTP (gamma glutamyl transpeptidase), male < 80 IU/L, female < 30 IU/L; T-Bil (total bilirubin), 0.2-1.1 mg/dL; Alb (albumin), 3.8-5.3 g/dL; collagen type IV, < 150 ng/mL.

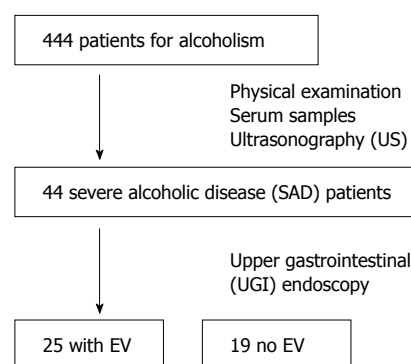


Figure 1 Flow chart of patients in this study. EV: Esophageal varice.

cance using the Mann-Whitney U test and a χ^2 test where appropriate. Independent predictive factors associated with esophageal varices were assessed by a multivariate analysis using a logistic regression model. The sensitivity and specificity of collagen type IV for predicting esophageal varices was determined using receiver operating characteristic (ROC) curves. A P value of less than 0.05 was considered to be statistically significant. All analyses were performed using the STATA 10.0 software program (STATA Corporation, College Station, Texas, USA).

RESULTS

Twenty-five patients (56.8%) had esophageal varices, and 19 (43.2%) had no varice (Figure 1). A univariate analysis revealed a significant difference between patients with and without esophageal varices with regard to age and type IV collagen levels (Table 1 and Figure 2).

These two variables that were significantly linked to the presence of esophageal varices in the univariate analysis, and a factor previously reported^[11], age, PT, and type IV collagen, were assessed by multivariate analysis. A logistic regression

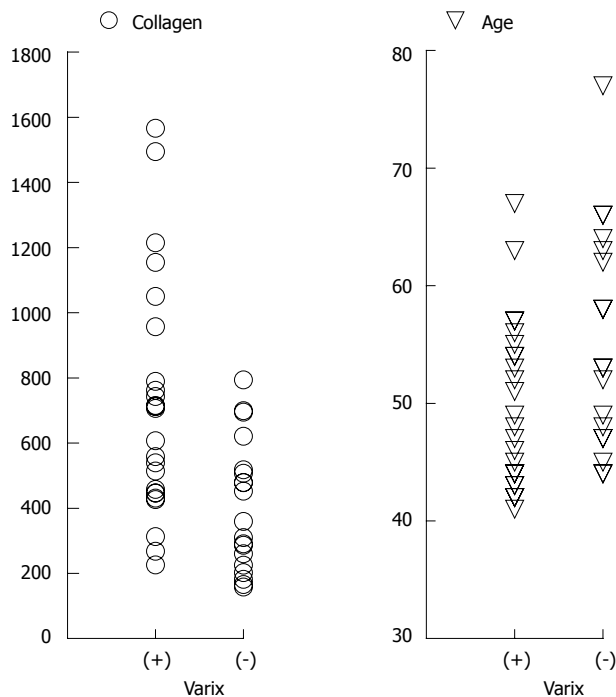


Figure 2 Two independent factors correlated with the appearance of esophageal varices. Collagen: Collagen type IV (ng/mL); (+): Positive patients; (-): Negative patients.

Table 2 An independent factor for the presence of esophageal varices (odds ratio)

Variable	Odds ratio	95% CI	P value
Collagen type IV	2.02	1.13-3.60	0.017

Data: Collagen type IV per 150 ng/mL.

analysis identified type IV collagen as the only independent variable predictive for esophageal varices ($P = 0.017$) (Table 2). Whenever the type IV collagen level raised every 150 ng/mL, the odds ratio of esophageal varices doubled.

Figure 3 shows the positive predictive values at each cut off point of type IV collagen. The positive predictive value of esophageal varices with a type IV collagen value > 900 ng/mL ($n = 6$) was 100%.

Finally, the area under the curve (AUC) of type IV collagen as determined by ROC for predicting the presence of esophageal varices was 0.78 (Figure 4).

DISCUSSION

A rupture of esophageal varices is the most frequent complication of portal hypertension, occurring in one third of all cirrhotic patients, and is associated with a high mortality^[12,13]. The mortality rate from variceal bleeding is about 20% when patients are treated optimally in a hospital^[14]. However, an appreciable proportion of patients with variceal bleeding die before reaching the hospital^[15]. Numerous studies have shown that the prevention of UGI bleeding and early detection of esophageal varices reduces mortality, morbidity, and health care costs^[16]. Nevertheless,

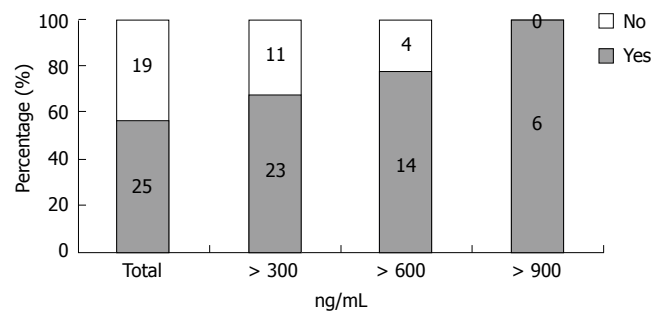


Figure 3 The positive predictive value for esophageal varices of collagen type IV. Y-axis: Positive percentages of esophageal varices; X-axis: The total predictive value of esophageal varices; > 300 (600 , 900): The positive predictive value of esophageal varices with a collagen type IV value > 300 (600 , 900) ng/mL.

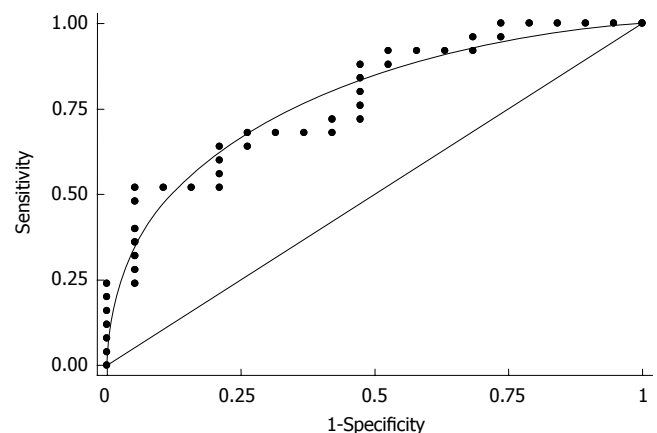


Figure 4 Receiver operating characteristics (ROC) curve of collagen type IV for the diagnosis of esophageal varices [area under curve = 0.7802, se (area) = 0.0704].

Suzuki *et al* demanded further studies to determine which strategies are the most beneficial to patients and society in terms of preventing and treating esophageal varices, in a recent article^[7]. We thus examined potential esophageal varice prediction factors for SAD on a medical checkup level.

In this study, a non-invasive marker for hepatic fibrosis (type IV collagen) had a high diagnostic accuracy for the detection of esophageal varices. The combination of abdominal ultrasound scan and this marker correctly identified, at a high rate, patients with esophageal varices. These examinations can be conducted on a medical checkup level, so we considered this approach to be of considerable diagnostic value.

A previous report showed the type IV collagen concentration was the most accurate in correctly identifying patients with severe histologic alcoholic hepatitis. At a cut-off of 150 ng/mL, type IV collagen was 89% sensitive and 77% specific^[17]. First, patient sorting was conducted via the abdominal ultrasound test in this study. The alcoholic patients with splenomegaly and/or withering of the right liver lobe participated in this study. In these 44 patients, the value of type IV collagen was > 150 ng/mL in all specimens. As a result, when suspecting SAD, we thought it very meaningful to add the abdominal

ultrasound test to the characteristics under evaluation.

In a previous report, Geoffroy and colleagues demonstrated that the independent factors of prothrombin index, alkaline phosphatase activity, and hyaluronate level predicted the presence of esophageal varices^[11]. Nevertheless, their proposed model included two age-dependent serum markers, hyaluronate and alkaline phosphatase, both of which rise in serum with aging^[18]. We therefore added only the prothrombin index as an examination item. Moreover, another report demonstrated a rise in amino-terminal procollagen III peptide (PⅢNP) following alcohol withdrawal that is likely to be caused by intact PⅢNP^[19]. We thus decided not to include this fibrosis marker as an examination item at the time of hospitalization. Finally, we elected to add GTP and collagen type IV as examination items.

The serum concentration of laminin and type IV collagen have been reported to be increased in patients with alcoholic hepatitis and to correlate with the degree of inflammation^[20-26]. In our study, logistic regression identified type IV collagen as the only independent variable predictive for esophageal varices. While based on the findings of these studies, laminin may be predictive for esophageal varices. However, the use of such testing is not covered by the national health insurance program in Japan, so we decided to exclude laminin from this study. We therefore hope that further study of esophageal varices in other countries will help to elucidate and confirm the predictive potential of laminin.

Antler *et al* found that in younger patients, the most common bleeding sites are those associated with alcoholism (esophageal varices, Mallory-Weiss tears, and hemorrhagic gastritis), accounting for 40%-60% of lesions in patients less than 55 years-of-age^[27]. Another report demonstrated that younger patients had a trend toward more variceal bleeds ($P = 0.39$)^[28]. In our research, there was a positive correlation of esophageal varices with younger age ($P > 0.05$). We think that younger alcoholic patients with high fibrosis markers should be evaluated by GI endoscopy.

In conclusion, this study suggests that the level of type IV collagen has a high diagnostic accuracy for the detection of esophageal varices in SAD. These results show that the non-invasive screening of patients who are at risk for variceal bleeding is possible, and that this approach may assist in the prevention of this most serious complication.

COMMENTS

Background

Bleeding from varices may occur in 15%-68% of patients with varices. Variceal hemorrhaging is associated with a high mortality rate, as well as high hospital costs.

Research frontiers

The early detection of esophageal varices is critical for the effective prevention of variceal hemorrhaging. Adding an accurate non-invasive diagnostic model may improve the diagnostic accuracy in predicting esophageal varices without performing liver biopsy.

Innovations and breakthroughs

First, patient sorting for alcoholism was conducted via the abdominal ultrasound

test in this study. The existence of esophageal varices with severe alcoholic disease was compared according to a number of clinical background variables. A univariate analysis revealed a significant difference in age and type IV collagen levels between the patients with and without esophageal varices. A logistic regression analysis identified type IV collagen as the only independent variable predictive for esophageal varices ($P = 0.017$). AUC of type IV collagen as determined by ROC for predicting expressed esophageal varices was 0.78.

Applications

The combination of an abdominal ultrasound scan and type IV collagen correctly identified, at a high rate, alcoholism patients with esophageal varices.

Terminology

The type IV collagen and laminin are the major components of basement membranes. Early accumulation of type IV collagen and laminin, thus leading to the formation of basement membrane-like material in the space of Disse (capillarization), is considered a typical characteristic of alcoholic liver disease. The amount of collagen in the space of Disse has been shown to correlate significantly with the presence of alcoholic hepatitis and portal blood pressure.

Peer review

This short paper summarized well the relevance of type IV collagen as a predictive factor for varices in alcoholic liver cirrhosis.

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Early effects of Lansoprazole orally disintegrating tablets on intragastric pH in CYP2C19 extensive metabolizers

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Abstract

AIM: To compare rabeprazole (RPZ; 10 mg) with Lansoprazole orally disintegrating tablets (LPZ; 30 mg OD) in terms of antisecretory activity and blood drug concentration after a single dose.

METHODS: Eight *H. pylori*-negative cytochrome P450 (CYP) 2C19 extensive metabolizers were assigned to receive a single oral dose of RPZ 10 mg or LPZ 30 mg OD. Twelve hour intragastric pH monitoring was performed on the day of treatment. Blood samples were also collected after the administration of each drug.

RESULTS: LPZ 30 mg OD induced a significantly earlier rise in blood drug concentration than RPZ 10 mg; consequently, LPZ 30 mg OD induced a significantly earlier rise in median pH in the third and fourth hours of the study.

CONCLUSION: In *H. pylori*-negative CYP2C19 extensive metabolizers, LPZ 30 mg OD induced a significantly faster inhibition of gastric acid secretion than RPZ 10 mg.

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Key words: LPZ 30 mg orally disintegrating tablets; Intragastric pH; Blood drug concentration; Cytochrome P450 2C19 extensive metabolizers; *H. pylori*-negative

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INTRODUCTION

Gastroesophageal reflux disease (GERD) is a common disease in the West^[1-3], with increasing prevalence in Japan^[4-7]. A recent study in Japan by Ohara *et al* has shown that a total of 42.2% of Japanese experienced heartburn^[6], which is a similar proportion to the estimated 42.4% reported in Western studies^[1]. Moreover, endoscopic studies have shown the overall prevalence of reflux esophagitis (RE) among the adult population of Japanese outpatients is 14%-16%^[4-7]. The mechanism of GERD is closely associated with gastric acid; thereby, gastric acid suppression is the most common therapeutic approach, and stronger and prompter gastric acid suppression is required^[8]. At present, drug therapy for reflux esophagitis is common because of its effectiveness; acid suppressing drugs such as H₂ receptor antagonist (H₂RA) and proton pump inhibitors (PPIs) are commonly used. As PPIs have been shown to be more effective against RE than H₂RA^[9-11], PPIs such as lansoprazole (LPZ) and rabeprazole (RPZ) are now widely used as first-line acid inhibitors. Continuous maintenance with PPIs is considered to be the mainstay of GERD treatment.

However, there are some reports not showing that all GERD patients need continuous acid inhibition. Bour *et al* reported that on-demand therapy with PPI provides an alternative to continuous therapy in patients with mild to moderate gastro-esophageal reflux^[12]. Several reports have

demonstrated that on-demand therapy with PPI provides an alternative to continuous therapy in patients with mild to moderate gastro-esophageal reflux^[13].

In Japan, many studies on RE have been done and although each report differs slightly, an obvious trend is apparent, showing most patients suffered from mild RE^[5-7], which is milder than that experienced in Western countries. As a result, some Japanese patients take PPIs when required according to their symptoms in the clinical setting.

It is reported that rapid acid suppression is important for effective pain relief at the onset of treatment in GERD patients^[14]. Thus, the aim of this study was to examine the correlation between pH value and blood drug concentration in patients treated with RPZ 10 mg or LPZ OD 30 mg at the early post-administration phase (1-12 h).

RPZ and LPZ are generally administered at doses of 10 mg or 30 mg, respectively, in the clinical setting in Japan. Thus, in the present study, the RPZ and LPZ doses were set at 10 mg and 30 mg, respectively.

Furthermore, in Japan many outpatients are administered multiple drugs. Hence, in order to improve drug compliance, RPZ and LPZ are also generally administered after a meal with other drugs. Therefore, we administered both RPZ and LPZ after a meal.

The acid-inhibitory effects of PPIs are significantly dependent on the cytochrome P450 (CYP) 2C19 genotype status, as well as on their intrinsic pharmacokinetic and pharmacodynamic characteristic and dosing schemes^[15-19]. According to these reports, the metabolism of PPIs is affected by the CYP2C19 polymorphism; the plasma PPI levels and intragastric pH values in extensive metabolizers are significantly lower than those in poor metabolizers^[20-22]. Therefore, in this study, the subjects were all CYP2C19 extensive metabolizers.

MATERIALS AND METHODS

Subjects

The subjects were 8 healthy male volunteers, aged between 24 and 48 years (median, 23 years) and weighing 52-78 kg (median, 54 kg). No patient had a history of gastrointestinal or hepatobiliary disease or of eradication therapy for *H pylori*, and none took regular medications. All volunteers gave written informed consent. The study protocol was approved by the ethical committee of the Tohoku University Graduate School of Medicine.

Detection of *H pylori* infection

H pylori infection was determined by the ¹³C-urea breath test^[23]. A total of 8 *H pylori*-negative subjects were invited and approved to participate in this study.

CYP2C19 genotyping

After obtaining informed consent, a venous blood sample was collected from all patients. DNA was extracted from the nucleus of venous white blood cells. The genetic mutation was analyzed by either the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method^[24] or the TaqMan polymerase chain reaction

amplification method (Applied Biosystems Japan, Chiba, Japan)^[25]. Based on point mutations in exons 4 and 5 of the CYP2C19 gene, individuals can be classified into homo-extensive metabolizers (homo-EMs), hetero-extensive metabolizers (hetero-EMs) and poor metabolizers (PMs)^[20-22]. Homo-EMs have the wild type alleles (wt/wt) without any mutations in exons 5 and 4; PMs have mutated alleles (m1/m2) with mutations in both exons 5 and 4 (m1/m2, m1/m1 or m2, m2); and hetero-EMs have a mutated allele in either exon 5 or 4 (wt/m1 or wt/m2).

Study protocol

All subjects (homo EM = 4, hetero EM = 4) participated in an open-label crossover study with RPZ 10 mg tablets or LPZ 30 mg OD. They were randomly assigned to receive a single oral dose of RPZ 10 mg tablet or LPZ 30 mg OD 30 min after eating a standardized meal. There was a washout period of at least 14 d between the two study periods. Twenty-four-hour intragastric pH monitoring was performed on the day of treatment. To monitor gastric pH, a pH electrode was inserted transnasally, and positioned fluoroscopically in the gastric corpus, approximately 5 cm-10 cm below the esophago-gastric junction. Gastric pH was measured at 10 s intervals by means of a portable pH meter attached to a glass pH electrode (Chemical Instrument, Tokyo, Japan). The pH electrode was calibrated before each recording, using standard buffers of pH 1.68, 4.01 and 6.86. The pH data were analyzed with the use of established software (Chemical Instrument). At fixed times (Breakfast 8:30 AM, lunch at noon, snack at 15:00 and dinner at 7:00 PM), standardized meals were consumed (total 1359 kcal; protein, 24 g lipid, 18.5 g glucose 267 g. The individual calorie contents of breakfast, lunch, snacks and supper were 356, 324, 355 and 324 kcal respectively). No additional food was allowed, and 100 mL of tap water was allowed only when the subjects felt thirsty. All subjects were instructed to remain upright until 21:00. Normal daily activities were not restricted.

Sample collection and assay of LPZ, RPZ concentration in plasma

In order to study the correlation between intragastric pH and blood drug concentration, blood samples were collected in heparinized tubes before and 0.5, 1, 1.5, 2, 3.5, 6, 9 and 12 h after the administration of each drug. After collection, the blood samples were immediately centrifuged at 3000 r/min for 10 min. For the determination of RPZ levels in plasma, 100 µL of 1% diethylamine solution was added to 1 mL of plasma; this was not required for the determination of plasma concentrations of LPZ. All samples were stored at -20°C until assayed. Plasma levels of LPZ and RPZ were measured by high-performance liquid chromatography/tandem mass spectrometry^[26,27]. This method required only 20 µL of serum and is a simple procedure. Analytes and the internal standard (lansoprazole deuterium derivatives) were separated using a mobile phase of acetonitrile/1 mmol/L ammonium formate (140/60, v/v) on a C18 analytical column and analyzed in the selected reaction-monitoring (SRM) mode. The lower limit of quantification was 500 fg/20 µL.

Table 1 Characteristics of the subjects in this study

Subject	CYP2C19	Age	Height (cm)	Body weight (kg)	BMI
1	Hetero	33	175	70	22.8
2	Hetero	50	175	73	23.8
3	Hetero	31	168	65	23.0
4	Hetero	22	165	60	22.0
5	Homo	31	168	65	23.0
6	Homo	26	173	68	22.7
7	Homo	23	171	70	23.9
8	Homo	25	184	75	22.1

Statistical analysis

Intragastric pH were expressed as median values (ranges). Differences in these parameters among each regimen were determined by the Wilcoxon signed rank test. *P* values less than 0.05 were considered significant.

RESULTS

Eight volunteers (all men; mean age 30.3 years, range years) completed the study. There were no adverse events during the study, which was completed according to the protocol by all 8 subjects. Four subjects were homo-EMs, and the other 4 subjects were hetero-EMs (Table 1).

The 12-h trendgram and the profiles of correlation between intragastric pH and blood drug concentration are shown in Figure 1. The lower limit for quantification of blood drug concentration was 500 fg/20 µL in this study. However, the blood drug concentration of RPZ was not detectable until 2 h after drug administration, while LPZ was detectable 0.5 h after drug administration. LPZ 30 mg OD induced a significantly earlier rise in blood drug concentration than RPZ 10 mg tablets.

As a result of this prompt rise in blood LPZ concentration, there was a prompt onset of median pH. The 12-h (median pH per hour) trendgrams of intragastric pH values obtained without medication are shown in Figure 1.

The intragastric pH values increased significantly with both drugs. LPZ 30 mg OD increased the pH value after the second hour of the study, while RPZ 10 mg tablets increased the pH value after the fourth hour of the study, compared with those pH values of individuals without medication.

LPZ 30 mg OD induced a significantly earlier rise in median pH in the third and fourth hours of the study than RPZ 10 mg tablets (Figure 1).

DISCUSSION

PPIs, such as omeprazole, LPZ and RPZ, are widely used for the treatment of acid-related diseases. The frequency of GERD has increased recently, because of increased average fat intake^[28], increased rates of obese patients^[29,30], and declining rates of *H. pylori* infection^[31,32]. GERD is a common disease in the West^[1-3] and appears to be increasing in prevalence in Japan^[4-7]. Recent endoscopic studies have shown overall prevalence of reflux esophagitis among the adult population in Japan is 14%-16%^[5-7]. Each report differs slightly, but an obvious trend is that

most patients suffer from mild RE^[5-7]. Moreover, it is reported that the incidence of atrophic gastritis in the general population is higher in Japan than in Western countries^[33-35], and that gastric acid secretion levels in the general population are lower in Japan than in Western countries^[33-36]. Thereby, some patients in Japan want administration of the drug on demand.

Patients with GERD mainly suffer of intermittent symptoms rather than continuous symptoms^[37]. On-demand therapy with PPIs is reported to provide an alternative to continuous therapy in patients with mild to moderate gastro-esophageal reflux disease suffering from frequent symptomatic relapse^[12].

It is important to use medicines that immediately ameliorate the clinical symptoms. Therefore, it is useful to administer antisecretory drugs which have a faster and stronger onset of pH rise in the stomach among patients with acid-related disorders.

Thus, we studied the effect in the early post-administration phase (1-12 h) of a single dosing of each PPI. In this study, LPZ 30 mg OD induced a significantly earlier rise in blood drug concentration than RPZ 10 mg tablets.

As a result of this prompt rise in blood LPZ concentration, LPZ 30 mg OD induced a significantly earlier rise in intragastric pH values compared with the values in individuals without medication after the second hour of the study, while RPZ 10 mg tablets induced an earlier rise in the fourth hour of the study. Moreover, LPZ 30 mg OD also induced prompt and stronger inhibition of gastric acid secretion than RPZ 10 mg tablets in the early post-administration phase, in the third and fourth hours following a single oral dose of treatment.

These results differ slightly from those of previous reports^[17-19,38,39] in which RPZ induced an earlier rise in intragastric pH than other PPIs. However, most previous studies examined the effects after administration of RPZ or other PPIs on days 3-7^[17-19,38,39], not at the early post-administration phase (1-12 h) of a single dose of treatment, whereas the present study examined the effect in this phase.

There are interindividual variations in the metabolism of PPI, resulting in differences in the acid-suppressing effect of each PPI^[17-19,38,39]. Each report differs slightly, but CYP2C19 genotype status is shown to influence gastric acid suppression by LPZ and most other PPIs. The metabolism of LPZ, OPZ and other PPIs is affected by the CYP2C19 polymorphism, and the plasma PPI level and intragastric pH values of EMs are significantly lower than those of PMs^[20-22]. On the other hand, several studies have demonstrated that after a dose of RPZ, intragastric pH is not affected by the CYP2C19 polymorphism on 3 d-7 d of treatment^[17-19,38,39]. However, Horai *et al.*^[15] reported the pharmacodynamic effects and pharmacokinetics of RPZ depend on the CYP2C19 genotype status on the first day after a single dose. As the present study is on the effect on the first day after a single dose of PPIs, both RPZ and LPZ are considered to be influenced by CYP2C19 genotype status.

Previous reports have indicated that *H. pylori* infection of the gastric mucosa potentiates the effects of proton pump inhibitors^[40,41]. Therefore, in the present study, the study subjects were all *H. pylori*-negative CYP2C19 EMs.

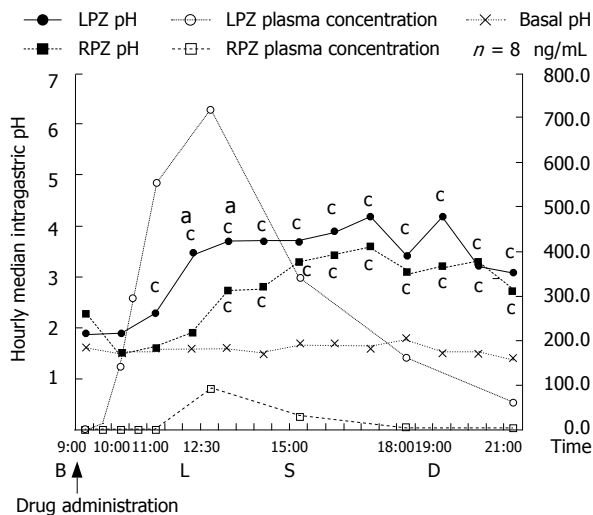


Figure 1 The 12-h (median pH per hour) trendgrams for all subjects ($n = 8$) and correlations with the blood concentration of each drug. The solid line (●) shows hourly intragastric median pH of individuals administered LPZ 30 mg OD, and the broken line (■) shows that of individuals administered RPZ. The solid line (○) shows the blood drug concentration level in individuals administered LPZ OD 30 mg, and the broken line (□) shows those in individuals administered RPZ 10 mg tablets. The solid line (x) shows the intragastric pH values of all subjects obtained without medication. LPZ 30 mg OD induces an earlier rise in both blood concentration level and median pH than RPZ 10 mg tablets. Arrows: Drug administration. B: Breakfast, L: Lunch, S: Snacks, D: Dinner. Blood sample were collected 1, 1.5, 2, 3.5, 6, 9 and 12 h after the administration of each drug. The significance of differences in these intragastric pHs among each regimen was determined by the Wilcoxon signed rank test. * $P < 0.05$ vs RPZ; † $P < 0.05$ vs baseline data.

Thus, we clearly state that LPZ 30 mg OD induces a significantly earlier rise in intragastric pH and stronger inhibition of gastric acid secretion than RPZ 10 mg tablets in the early post-administration phase (1-12 h) of a single dose of treatment.

Pipkin *et al* reported that rapid acid suppression is important for effective pain relief at the onset of treatment in GERD patients^[14]. Thereby, our results perhaps show that administration of LPZ 30 mg OD as an on-demand therapy is useful for mild GERD patients, because of its faster onset of pH rising action.

So why does LPZ 30 mg OD induce a prompt rise in intragastric pH than RPZ 10 mg tablets? This may be partly because of the difference between the dosage forms of RPZ and LPZ. In this study, we compared an enteric-coated tablet formulation of RPZ 10 mg tablets with an enteric-coated microgranule formulation of LPZ OD 30 mg in terms of antisecretory activity and the onset of action of a single dose.

PPIs are degenerated by gastric acid; therefore, for immediate passage through the stomach, some PPIs are formulated as granules or microgranules. This is necessary to ensure their intact passage through the stomach to allow for absorption in the intestine. The discharge speed, namely passage over time through the stomach, depends upon the particle diameter^[42-45].

According to physiological reports^[42-46], complexes of high amplitude action potentials occur in the stomach and duodenum. The interdigestive complex in the dog is looked upon as a “housekeeper”, which sweeps the

bowel clear of contents at the end of the digestive phase. Using a test food labeled with radionuclide, Davis SS *et al*^[46] reported that food emptied into the duodenum immediately, consisting of particles smaller than 2 mm without a “housekeeper”. Moreover, particles larger than 2 mm emptied into the duodenum after the “housekeeper,” which occurs after all meals have emptied from the stomach.

As a result, particles smaller than 2 mm empty from the stomach faster than particles larger than 2 mm. LPZ OD particles are smaller than 2 mm; thereby they may passage through the stomach into the duodenum and small intestine faster than RPZ, which is larger than 2 mm. They are absorbed in the small intestine and reach the gastric parietal cells *via* systemic circulation, where they bind to the proton pump, thereby resulting in potent acid inhibition^[16]. In fact, the plasma concentration level of LPZ 30 mg OD induced a prompt effect than RPZ 10 mg tablets, and consequently, LPZ 30 mg OD induced a prompt rise in intragastric pH than RPZ 10 mg tablets. These findings suggest that LPZ 30 mg OD is suitable for administration as an on-demand PPI, because of the prompt rise in plasma concentration level and the faster rise in intragastric pH.

In conclusion, LPZ 30 mg OD induced a significantly earlier rise in plasma concentration level in the early post-administration phase of a single oral dose than RPZ 10 mg tablets. As a result, LPZ 30 mg OD induced a significantly earlier rise in median pH in the early post-administration phase of a single oral dose than RPZ 10 mg tablets.

COMMENTS

Background

The prevalence of gastroesophageal reflux disease (GERD) symptoms is now increasing in Japan. GERD has a high rate of relapse. The rising use of proton pump inhibitor (PPI) therapy on demand has raised issues regarding efficacy.

Research frontiers

To compare RPZ 10 mg to LPZ 30 mg OD in terms of their antisecretory activity and blood drug concentration in the ultra-early phase after a single dose.

Innovations and breakthroughs

Most previous studies have examined pH monitoring after administration of PPI on days 3-7, not at the early post-administration phase.

Applications

We clearly state that LPZ 30 mg OD induced a significantly earlier rise in intragastric pH and stronger inhibition of gastric acid secretion than RPZ 10 mg tablets during the early post-administration phase (1-12 h) of a single dose of treatment. It is reported that rapid acid suppression is important for effective pain relief at the onset of treatment in GERD patients. Thereby, our results show administration of LPZ 30 mg OD as an on-demand therapy might be useful for mild GERD patients because of its faster onset of pH rising action.

Peer review

In this manuscript, the authors ascertained the effectiveness of LPZ 30 mg OD compared with RPZ 10 mg in the elevation of intragastric pH in the ultra-early state after a single oral administration. The study was well performed and the conclusion was clinically useful.

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***p16* promoter hypermethylation: A useful serum marker for early detection of gastric cancer**

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methylation status of *p16* promoter. *p16*-protein expression was analyzed by immunohistochemical staining on paraffin-embedded sections.

RESULTS: Methylation was detected in 44.2% (23/52) of tumoral tissues. 60.9% of them were also methylated in serum, i.e., 26.9% of all patients (14/52). Methylation was not detected in tissue and sera of control samples. *p16*-protein expression was decreased in 61.5% of cases (32/52), and was significantly associated with promoter hypermethylation ($P < 0.001$). Methylation was significantly more frequent in higher pathological grades ($P < 0.05$). Methylation was not associated with other clinicopathological features and environmental factors including *H pylori* infection and smoking.

CONCLUSION: *p16* promoter hypermethylation is an important event in gastric carcinogenesis. It is the principle mechanism of *p16* gene silencing. It is related to malignant tumor behavior. Detection of DNA methylation in serum may be a biomarker for early detection of gastric cancer.

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Key words: Gastric cancer; *p16*; Hypermethylation; Methylation specific PCR

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Abstract

AIM: To determine *p16* promoter hypermethylation in gastric tumoral tissue and serum samples, its impact on *p16*-protein expression, and correlation with clinical and histological features.

METHODS: Samples were obtained from 52 histologically confirmed cases of gastric adenocarcinoma. Gastric tissue and serum of 50 age- and sex-matched individuals with normal gastroscopy and biopsy were obtained as control samples. Methylation-specific polymerase chain reaction (MSP) was used to evaluate

INTRODUCTION

Gastric cancer is one of the most widespread cancers and the second leading cause of cancer-related death worldwide^[1]. It is estimated that gastric cancer is the most common cancer in Iran (Age Standardized Rate = 26.1 per 10⁵), and the incidence rate is higher than the world

average^[2]. Several genetic and epigenetic alterations have been suggested to play important roles in the carcinogenesis pathway, affecting oncogenes, tumor suppressor genes, apoptosis-regulating or mismatch repair genes^[3]. Repression of genes by CpG island methylation in the promoter region, which is normally unmethylated, is the most frequent epigenetic alteration, in which the DNA structure is affected while the genetic code remains intact^[4]. Thus, an increasing number of genes, methylated at the promoter region, are targeted as possible tumor markers for different purposes such as early detection, classification and tumor prognosis, therapeutic strategies and patient follow up^[5-9].

Detection of circulating tumoral DNA was first reported about three decades ago^[10]. Free DNA is thought to originate from apoptotic and necrotic tumoral cells^[11]. More recently, detection of promoter hypermethylation in serum of patients has been reported in some malignancies such as colorectal or esophageal cancer^[12,13]. Correlation of clinicopathological features with methylation patterns, which help to predict patient outcome, has been indicated in several studies^[14-18].

p16 is a cell-cycle regulator that induces G1-phase arrest by inhibition of cyclin D-dependent protein kinase 4 (CDK4) and 6 (CDK6), thus interfering with phosphorylation of the retinoblastoma protein (pRb) and further inhibition of transcription of proteins that promote passage of the cell through the restriction point of the G1 stage^[19]. *p16* inactivation breaks down the regulatory mechanism of the cell cycle. As a tumor suppressor gene, being silenced by any mechanism will promote carcinogenesis. This study was conducted to assess the methylation of *p16* promoter in gastric tumoral tissue and serum samples and its impact on gene expression, and correlation with clinical and histological features for the first time in the Iranian population.

MATERIALS AND METHODS

Sample collection and DNA preparation

Tumoral tissue and corresponding serum samples were obtained from 52 consecutive histologically confirmed gastric adenocarcinoma patients. Patients undergoing any therapeutic intervention were excluded. Tumoral tissues were obtained by gastrectomy or endoscopy in unresectable metastatic cases, formalin-fixed and paraffin-embedded. Tumors were histologically verified as gastric adenocarcinoma and subtyped into intestinal, diffuse, or mixed type, as suggested by Lauren^[20]. Grading was also determined and staging was performed using tumor, node and metastasis (TNM) classification, sixth edition. Fifty age- and sex-matched individuals, with normal gastroscopy and biopsy, were included as a control group. Paraffin-embedded tissue and corresponding serum samples were taken as well. All patients and control individuals gave informed consent according to institutional guidelines, and the study was approved by the research ethics committee of Mashhad University of Medical Sciences. Paraffin-embedded tissues were retrieved by using xylene and alcohol, digested by proteinase K, extracted with phenol/chloroform/isoamyl alcohol, and precipitated in ethanol. Serum samples were isolated by DNA extraction

Table 1 Primer sets used in MSP

Primer sets	Sense primer: 5'-3'	Antisense primer: 5'-3'	Size (bp)
p16-W	CAGAGGGTGGGG CGGACCGC	CGGGCCGCGGCCGTGG	140
p16-M	TTATTAGAGGGTG GGGCGGATCGC	GACCCCGAACCGCGAC CGTAA	150
p16-U	TTATTAGAGGGTG GGGTGGATTGT	CAACCCCAAAACCACAA CCATAA	151

W: Unmodified or wild-type primers; M: Methylated-specific primers; U: Unmethylated-specific primers.

kit (QIAamp DNA mini kit, QIAGEN, Canada). *H. pylori* infection was detected and double checked in serum with an ELISA (Trinity *H. pylori* kit, Ireland). None of the patients had a history of *H. pylori* eradication.

DNA modification (bisulfite treatment)

DNA modification with sodium bisulfite causes unmethylated cytosine bases to convert to uracil, while methylated cytosine is resistant and remains unchanged. After treatment, methylated alleles have a different sequence as compared with unmethylated alleles, which can be used to design allele-specific PCR primers, and methylation-specific PCR (MSP) takes advantage of this fact^[21]. Two micrograms of genomic DNA was first denatured by heating (97°C for 10 min followed by chilling on ice at 0°C for 5 min), and incubated for 20 min at 48°C after adding 3 mol/L NaOH (2 µL). Bisulfite solution (2.5 mol/L sodium metabisulfite and 125 mmol/L hydroquinone) was added and incubated for 12 h at 48°C in the dark, for modification. Modified DNA was purified by using Wizard DNA purification resin (DNA Cleanup Kit; Promega, Madison, WI, USA) according to the manufacturer's instructions. Modified DNA was treated with 3 mol/L NaOH (5 µL) in 37°C for 10 min and precipitated with ammonium acetate 5 mol/L (75 µL), 2.5 volumes 100% ethanol and 2 µL glycogen (20 mg/mL; Fermentase; UAB, Lithuania) and dissolved in 20 µL 5 mmol/L Tris (pH 8.0).

MSP

Specific primer sets for unmethylated (p16U) and methylated (p16M) DNAs, described by Herman *et al*^[21], were utilized (Table 1). The PCR mixture contained 1 × buffer (Finzymes OY, Finland) with 2 mmol/L MgCl₂, 500 nmol/L each primer, 0.2 mmol/L dNTPs, 1 U Hot Start Taq polymerase (Finzymes OY, Finland). The PCR amplification of the modified DNA samples consisted of one cycle of 95°C for 10 min, 40 cycles of 94°C for 45 s, 60°C for 45 s, and 72°C for 1 min; then one cycle of 72°C for 10 min. DNA from L132 (embryonic lung cell line) cells was used as a positive control for unmethylated DNA. In order to make a positive control for methylated DNA, normal lymphocyte DNA was treated with M.Sss1 CpG methyltransferase (New England BioLabs, USA) before bisulfite treatment. Six microliters of amplified PCR products were loaded onto 2.5% agarose gels and non-denaturing 8% polyacrylamide gels, stained with ethidium bromide, and directly visualized under UV illumination.

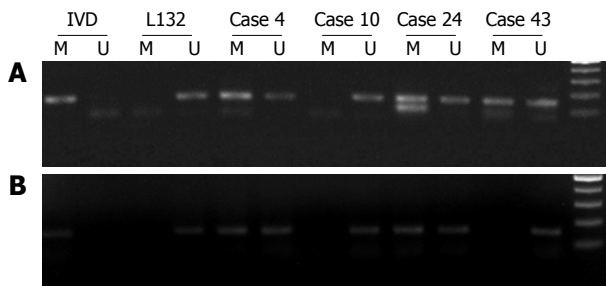


Figure 1 Analysis of *p16* promoter hypermethylation in tissue and corresponding serum of patients. **(A)** MSP analysis. IVD served as a positive control for hypermethylated DNA and L132 as a positive control for unmethylated DNA. Patients 4, 24 and 43 were hypermethylated, which revealed 150 bp bands with hypermethylated primers. Patient 10 was not methylated. **(B)** MSP analysis in corresponding sera of samples depicted in **A**. Patients 4 and 24 were hypermethylated in serum as well. Patients 43 and 10 were not methylated.

Table 2 Results of methylation in tumoral tissue and corresponding serum

	Tissue (+)	Tissue (-)
Serum (+)	14	0
Serum (-)	9	29

(+): Methylated; (-): Unmethylated.

Immunohistochemical staining

Immunohistochemical staining was performed using the CINtec p16^{INK4A} Histology Kit, clone E6H4 (Dako, Carpinteria, CA, USA) and the DakoCytomation Autostainer Instrument, according to the manufacturer's instructions. Briefly, 4- μ m-thick formalin-fixed, paraffin-embedded sections were dewaxed, rehydrated and boiled in Target Retrieval Solution of Dako in a microwave oven for 40 min. After endogenous peroxidase blocking, the slides were incubated with primary p16^{INK4A} antibody (clone E6H4) at 1:25 dilutions for 30 min. The antigen-antibody reaction was visualized by 3,3'-diaminobenzidine (DAB) chromogen for 10 min, followed by acidified hematoxyline counterstaining for 1 min. p16-positive cervical squamous cell carcinoma was used as an external positive control, and non-neoplastic stromal cells served as internal positive controls for *p16* in every tumor section.

Statistical analysis

Statistical analysis was performed using SPSS software (ver. 11.5). The correlation between two variables was evaluated using Pearson's χ^2 and Fisher's exact test. Statistical significance was defined as $P < 0.05$.

RESULTS

The studied population consisted of 38 men and 14 women. The median age was 64.5 and the average was 63.7 years (range, 38-81). Hypermethylation of *p16* promoter was detected in 44.2% of tumoral gastric tissue (23/52), while normal gastric samples were all unmethylated. MSP analysis of the *p16* promoter in gastric cancer is shown in Figure 1. Corresponding serum samples were also examined. Among the patients with methylated gastric

Table 3 Clinicopathological features of *p16* promoter hypermethylation

Variable	n (%)	Methylated	Unmethylated	P value
Total		23	29	
Gender				
Male	38 (73.1)	17	21	0.904
Female	14 (26.9)	6	8	
Age (yr)				
< 64	26 (50.0)	8	18	0.051
> 64	26 (50.0)	15	11	
Pathological grade				
1	20 (39.2)	5	15	< 0.05
2	11 (21.6)	7	4	
3	20 (39.2)	10	10	
Pathological type				
Intestinal	27 (62.7)	13	14	0.754
Diffuse	14 (27.5)	5	9	0.161
Mixed	5 (9.8)	4	1	0.140
Anatomical site				
Cardia	22 (44.0)	7	15	0.124
Body	15 (30.0)	9	6	
Pylorus	13 (26.0)	6	7	
Distant metastasis				
Absent	39 (75.0)	20	19	0.259
Present	13 (25.0)	9	4	
Smoking				
Yes	9 (17.6)	4	5	1.000
No	42 (82.4)	19	23	
<i>H. pylori</i> infection				
Positive	30 (60.0)	11	19	0.201
Negative	20 (40.0)	11	9	

tissue, 60.9% were also methylated in their corresponding serum, i.e., 26.9% of all cases (14/52). All normal individuals in the control group were unmethylated in their sera (Table 2).

Clinicopathological features of *p16* promoter hypermethylation are tabulated in Table 3. There was no association between gender and promoter hypermethylation. Methylation had higher frequency in older patients ($P = 0.051$). Methylation was significantly lower in well-differentiated tumors as compared with higher pathological grades ($P < 0.05$). There was no significant difference in methylation status between intestinal and diffuse types. However, it was observed that 4/5 mixed type tumors were methylated, and all five were negative for p16 protein expression. Methylation was more frequent in non-cardiac type of tumor as compared with cardiac type (68.2% *vs* 31.8%), without statistical significance. *p16* promoter methylation had no correlation with distant metastasis. No association was observed between methylated circulating DNA and lymph node metastasis.

Among environmental factors, we assessed *H. pylori* infection and smoking. Sixty percent of patients (30/50) were infected with *H. pylori* and 17.6% (9/51) were smokers. *p16* promoter methylation had no correlation with smoking and *H. pylori* infection.

Immunohistochemical staining of p16

Nuclear p16 immunostaining was positive for protein

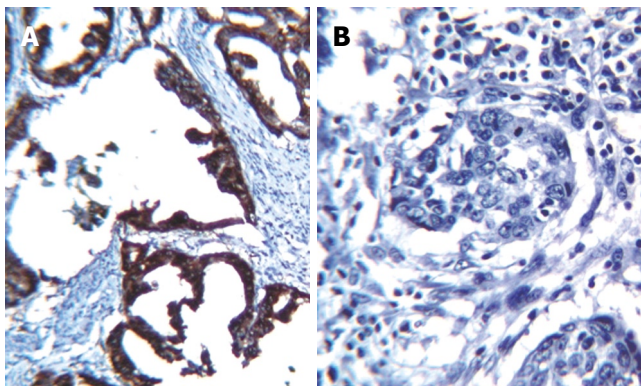


Figure 2 Immunohistochemical staining with monoclonal anti-p16 protein. (A) Nuclear reactivity showed expression of p16 protein (case 48). (B) p16-negative tumor (case 15) failed to stain due to decreased expression of p16 protein.

expression. Positive tumors varied in intensity of nuclear staining, with the proportion of cells ranging from 10% to 95%. In a few positive tumors, we observed marked heterogeneity in different areas with respect to p16 reactivity. Negative staining was observed in 61.5% of patients (32/52). Immunohistochemical staining is depicted in Figure 2. We assessed the correlation between immunohistochemical staining and methylation status (Table 4). Among the p16-negative tumors, 62.5% were methylated, which showed strong correlation between negative immunostaining and promoter region hypermethylation ($P < 0.001$). There was significant correlation with pathological grade and mixed subtype, as well as methylation pattern, but no association was observed with other clinicopathological and demographic features and environmental factors.

DISCUSSION

Several genetic and epigenetic alterations play an important role in gastric carcinogenesis. Tumor suppressor and other tumor-related genes are the main targets. Aberrant alterations of *p16*, as a tumor suppressor gene, are important events in several tumors, including gastric cancer, and hypermethylation of CpG islands in the promoter region is responsible for a great proportion of tumors^[22-25]. In this study, we demonstrated that 44.2% of tissues from gastric adenocarcinoma were methylated. The result was consistent with previous studies by Shim *et al*^[26] and Ding *et al*^[27] who reported 42% and 45% methylation of *p16* promoter in gastric cancer.

Different mechanisms are suggested for decreased p16 protein expression, including homozygote deletion, point mutation and promoter methylation^[28]. The first two mechanisms occur in $< 10\%$ of tumors^[29,30]. We report that 61.5% of gastric adenocarcinomas were negative for p16 protein expression. Among p16-negative tumors, 62.5% were methylated in their promoter region, which was strongly correlated with decreased protein expression. We conclude that the principle mechanism for decreased p16-protein expression in gastric cancer is hypermethylation of the promoter region. There were three patients with normal protein expression despite promoter hypermethylation. All of these exhibited tumor heterogeneity, although

Table 4 Results of methylation analysis in p16-positive and negative cases

Immunohistochemistry	Methylated	Unmethylated
p16-positive	3	17
p16-negative	20	12

partial methylation in one or both alleles might have been another possible reason. There were also twelve p16-negative patients with normal methylation status. Alternative mechanisms such as point mutation or homozygote deletion might be responsible for decreased protein expression.

Promoter hypermethylation was detected from sera of 60.9% of patients with tumor methylation, which accounted for 26.9% of all cases. This was similar to the study performed by Koike *et al*^[31] who reported 27% methylated sera in gastric cancer patients. There are some interesting features that introduce *p16* hypermethylation as a good serum marker for early detection of gastric cancer. As compared with other methods, serum markers are easier to use, less expensive and less invasive. Among serum markers, hypermethylation has some distinct features. It is a frequent event in cancer, while rare or absent in normal tissue, which leads to high specificity for the purpose of tumor diagnosis. Small specific regions of genome are affected, which make it easily detectable, in contrast with mutations. MSP is a sensitive technique that detects free circulating DNA even in small amounts^[32]. Among many genes altered by methylation in gastric cancer, *p16* promoter methylation is an early event in carcinogenesis^[33]. We conclude that *p16* is a good serum marker for early detection of gastric cancer.

In this study we showed that hypermethylation is less frequent in well-differentiated tumors. We conclude that *p16* promoter hypermethylation is associated with tumor malignant behavior. There were no significant differences between intestinal and diffuse types in our population, but mixed subtype was strongly associated with silenced expression. However, there were only five cases with mixed type among our patients, and additional studies with a larger number of cases are needed to confirm the finding. In the population studied, 86.5% were at stage 3 and 4, which showed that gastric cancer was diagnosed in advanced stages, which necessitates early detection. We did not statistically compare patients at different stages, because of unbalanced distribution and the consequent bias. Methylation status in cardiac tumors was also compared with that of non-cardiac tumors. We know that cardiac tumors have different behavior from non-cardiac tumors. Methylation tends to occur more in distal tumors (more than two-fold higher), without statistical significance. We did not find any association between *H. pylori* and methylation in our population, despite a few previous studies that suggested *H. pylori* induces methylation^[34,35]. Smoking, the other studied environmental factor, had no correlation with promoter hypermethylation. However, our patients consisted of only 17.6% (9/51) smokers, which was not sufficient to draw any strong conclusion. Promoter hypermethylation was not affected by gender, although it tended to occur more often in older patients. Precise

matching and absence of promoter hypermethylation in the control individuals decreased the possibility of methylation induction by aging, unrelated to cancer. No association was observed between circulating methylated DNA and lymph node metastasis. This indicates that the origin of circulating methylated DNA is not from lymph node metastasis. Hypermethylation status in serum was not associated with other clinicopathological features either.

In conclusion, *p16* promoter hypermethylation is an important event in gastric carcinogenesis. It is the principle mechanism of *p16* gene silencing. It is associated with tumor malignant behavior. Detection of *p16* hypermethylation in serum may be a useful biomarker for early detection of gastric cancer. Assessment of other serum markers may increase the sensitivity of screening in future studies. Moreover, promoter hypermethylation will regress after treatment. Further studies will determine whether promoter hypermethylation is a good surveillance marker for patient follow-up after treatment.

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COMMENTS

Background

Gastric cancer is the second leading cause of cancer-related death worldwide. This is because most cases are diagnosed at an advanced stage, and there is no standard method for early detection. Several genetic and epigenetic alterations play important roles in gastric carcinogenesis. Methylation, the most important epigenetic alteration, influences gene repression without affecting genetic coding. Several recent studies have been conducted to assess the clinical implications of epigenetics in cancer. Free circulating, tumor-derived DNA is a good target for early detection as a serum marker. *p16* is a tumor suppressor gene, which acts as a cell-cycle regulator. It is silenced in early stages of gastric carcinogenesis.

Research frontiers

P16 promoter hypermethylation related to clinicopathological features and early detection of gastric cancer.

Innovations and breakthroughs

A thorough analysis of *p16* promoter hypermethylation, correlations with demographic and clinicopathological features and environmental factors, and implications as an early diagnostic marker were performed for the first time in the Iranian population.

Applications

Although *p16* promoter hypermethylation is very specific for early detection of gastric cancer, further assessment of other genes will be useful to raise the sensitivity and provide a panel of serum markers for the purpose.

Peer review

This was a very interesting study. It examined *p16* promoter hypermethylation in gastric adenocarcinoma and free DNA. Methylation was detected in 44% of tumors, and in 26.9% of serum. *p16* promoter hypermethylation in serum was closely correlated with that in gastric tissue. Methylation was significantly associated with pathological grade.

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Prospective evaluation of small bowel preparation with bisacodyl and sodium phosphate for capsule endoscopy

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Abstract

AIM: To determine the effect of Prepacol[®], a combination of sodium phosphate and bisacodyl, on transit and quality of capsule endoscopy (CE).

METHODS: Fifty two consecutive patients were included in this prospective study. CE was performed following a 12 h fasting period. Twenty six patients were randomized for additional preparation with Prepacol[®]. The quality of CE was assessed separately for the proximal and the distal small bowel by 3 experienced endoscopists on the basis of a graduation which was initially developed with 20 previous CE.

RESULTS: Preparation with Prepacol[®] accelerated small bowel transit time (262 ± 55 min vs 287 ± 97 min), but had no effect on the quality of CE. Visibility was significantly reduced in the distal compared to the proximal small bowel.

CONCLUSION: The significantly reduced visibility of CE in the distal small bowel allocates the need for a good preparation. Since Prepacol[®] has no beneficial effect on CE the modality of preparation and the ideal time of application remains unclear. Further standardized examinations are necessary to identify sufficient preparation procedures and to determine the impact of the volume of the preparation solution.

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Key words: Small bowel; Capsule endoscopy; Preparation;

Laxative; Visibility; Transit time

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INTRODUCTION

Capsule endoscopy (CE) is a well accepted tool for evaluation of small bowel pathologies^[1-4]. However, it has some limitations due to restricted recording time and reduced visibility by air and residual material especially in the distal small bowel. Therefore, prokinetic drugs, laxatives and defoaming agents have been tested to improve the quality of the examination.

Prokinetic drugs were used to avoid incomplete small bowel examinations due to long gastric retention and slow bowel transit of the capsule. It was shown that domperidone shortened gastric emptying time of the capsule^[5]. The results on metoclopramide were inconsistent: Keuchel *et al*^[6] found no effect, whereas Selby^[6] demonstrated an increased gastric emptying time. Erythromycin accelerated gastric emptying^[7,8], however, this treatment had no effect on the visibility in one study^[7] and led even to an impaired visibility in another study^[8].

In order to clean the small bowel from residual material different laxatives were tested. Sodium phosphate improved the view in some studies^[9-11]. Preparation with polyethyleneglycol produced controversial results: In two studies the visibility was improved^[12,13], whereas in others it was unchanged^[14-16].

Preparation with simethicone, a defoaming agent, resulted in fewer air bubbles and better visibility in one study^[17].

However, since the data is scanty and partially inconsistent, to date no standardized protocol has been recommended for bowel preparation for CE.

Prepacol[®] (Guerbet GmbH, Sulzbach, Germany) is a combination of a saline (sodium phosphate) and a stimulant laxative (bisacodyl). It consists of 30 mL of a sodium phosphate solution (containing 6.9 sodium

monohydrogenphosphatedodecahydrate and 16.4 mg sodiumdihydrogenphosphatedihydrate) and 4 tablets (5 mg bisacodyl each). Prepacol® is available in several European countries and mainly applied for preparation before gastrointestinal operations, radiological and endoscopic bowel examinations.

The sodium phosphate solution is poorly absorbable. Water absorption from the gut is therefore impeded by the osmotic gradient. Besides its effects on colonic motor and secretory function, bisacodyl changes the net absorption of sodium and water in the small bowel into a net secretion^[18] and accelerates small intestinal transit^[18,19]. It was shown that bisacodyl elicits propulsive contractions of the terminal ileum^[20,21]. The combination of the osmotic purgative effect of sodium phosphate with the secretory and prokinetic effect of bisacodyl makes Prepacol® at least theoretically an ideal candidate for small bowel preparation for CE. However, its effect on the quality and gastrointestinal transit of CE has not been studied yet.

MATERIALS AND METHODS

Fifty two consecutive patients receiving capsule endoscopy were included. The patients were prospectively randomized into two groups. Group A fasted at least 8 h before the examination; group B received additionally Prepacol®. A written informed consent was obtained from all patients. The research protocol was approved by the ethics committee of the University of Heidelberg. All patients fasted from 7 p.m. the day before CE, patients in group B received additionally at 7 p.m. 30 mL of the sodium phosphate-solution diluted with 70 mL of tap water. Subsequently, they drank 250 mL of water. At 10 p.m. the patients received 4 tablets Prepacol® (20 mg Bisacodyl totally) again with 250 mL of water. All patients were allowed to drink water until 2 h before the examination. The capsule was swallowed at 10 a.m. with 250 mL of plain water.

Capsule endoscopy films were evaluated by three independent, endoscopically experienced investigators who were blinded concerning the kind of preparation. In a run-in-phase the three investigators corporately generated the appraisal factors and their graduation on the basis of 20 retrospective CE examinations. The following parameters were assessed: total quality of the film, visibility of small bowel mucosa, velocity of the capsule and occurrence of foam, air and residual food. Every parameter was graduated from 1 to 4, accordingly, excellent, good, limited and poor quality. Graduation for occurrence of foam is shown in Figure 1. To evaluate the effect of the preparation with Prepacol® two one-hour-lasting periods were evaluated. The first period started one hour after the capsule left the stomach, the second period ended when the capsule reached the ileocecal valve. Investigators examined the films at a rate of 20 pictures per second.

Quantitative data are expressed as mean \pm SEM and were analyzed by student's *t*-test for significant differences. Categorical data were evaluated by Chi² and Fisher exact test. *P* < 0.05 was chosen as the level of statistical significance.

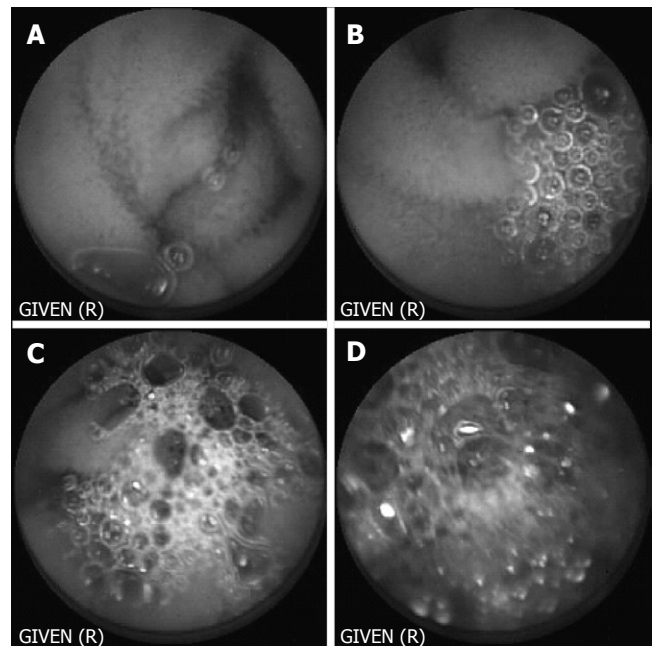


Figure 1 Graduation of visibility concerning occurrence of foam. A: Excellent; B: Good; C: Limited; D: Poor visibility.

Table 1 Patient's data and indications for CE: Group A (fasting) and group B (additionally Prepacol®)

	Group A	Group B	P value
Gender (w/m)	13/13	10/16	NS
Age (yr)	54 \pm 17	56 \pm 20	NS
Weight (kg)	71 \pm 15	79 \pm 17	NS
Length (cm)	170 \pm 10	171 \pm 8	NS
Indication			
GI-bleeding	17 (65%)	19 (73%)	NS
Inflammatory bowel disease	4 (15%)	3 (12%)	NS
Miscellaneous	5 (19%)	4 (15%)	NS

Data are mean \pm SEM; NS: Not significant.

RESULTS

Both groups were not different concerning age, weight, length and gender of the patients. Obscure gastrointestinal (GI) bleeding was the main indication for CE in both groups (Table 1). Other indications were suspicion for or follow-up in IBD, celiac disease, small bowel polyps or malignancy and no difference was observed between the groups (Table 1).

There were no differences in gastric emptying time of the capsule between the two groups (Table 2). Small bowel transit time was slightly but significantly shorter in the Prepacol®-group (262 \pm 55 min *vs* 287 \pm 97 min, *P* = 0.05) (Table 2). Recording time was not different between both groups.

Figure 2 demonstrates median assessment of investigators concerning visibility of small bowel mucosa, occurrence of foam, air and residual food, as well as velocity of the capsule and total quality of the film separately for the proximal and distal small bowel. Compared to exclusive fasting additional preparation with Prepacol® did not improve any parameter.

Table 2 GI transit times: Group A (fasting) and group B (additionally Prepacol®)

	Group A	Group B	P value
Gastric retention (min)	38 ± 23	44 ± 47	NS
Small bowel transit (min)	287 ± 97	262 ± 55	0.05
Total recording time (min)	441 ± 36	424 ± 49	NS

Data are mean ± SEM; NS: Not significant.

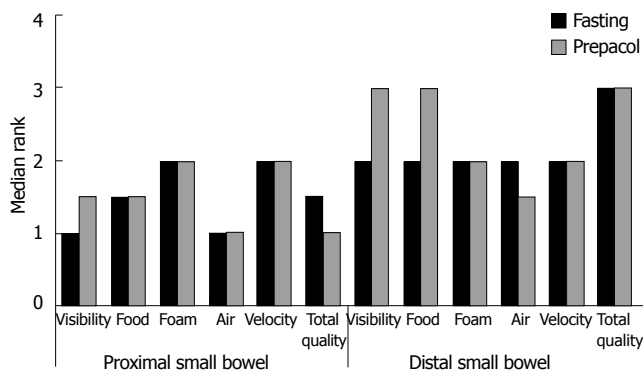


Figure 2 Quality of CE as assessed by three independent investigators, separately shown for proximal and distal small bowel, data are median of 52 patients, $n = 26$ for each group.

The quality was significantly more frequently judged as excellent or good in the proximal compared to the distal small bowel (Figure 3).

Concordance in the assessment between each of the investigators was good (82%, 78% and 87%, respectively).

DISCUSSION

An adequately cleaned bowel is an important precondition for any gastrointestinal endoscopic procedure. Turbid fluid due to intestinal secretion and food residues in the small bowel may limit visibility and therewith the information obtained by capsule endoscopy. Although in some studies preparation with prokinetic agents or laxatives improved quality of CE^[5-16], no standard procedure has been reached since the data is scanty and partially inconsistent. The selection of an appropriate preparation is further aggravated by the fact that the evaluation of the quality of capsule endoscopy is subjective. Therefore we chose three independent investigators to estimate the influence of Prepacol® on the quality of CE.

In the present study preparation with Prepacol® had no advantages concerning visibility of the mucosa, occurrence of foam, frequency and extent of air filled segments, food residues, velocity of the capsule and total quality as compared to exclusive overnight fasting.

Quality was significantly inferior in distal small bowel segments compared to proximal segments. This demonstrates that sufficient preparation would be of great help to obtain best possible conditions throughout the whole small bowel.

Prepacol® is not effective as a preparation for capsule endoscopy. This may be due to the pharmacological effect,

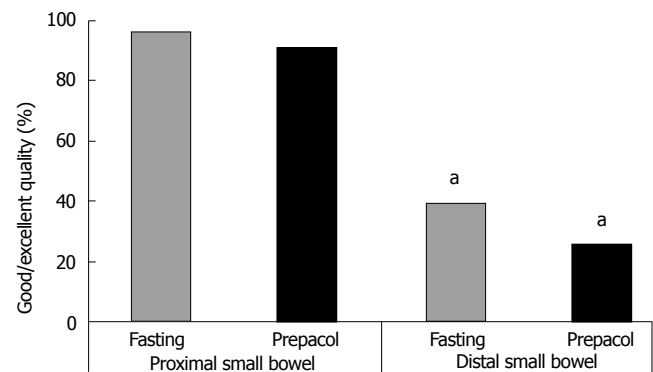


Figure 3 Percentage of good or excellent quality as assessed by three independent investigators, separately shown for proximal and distal small bowel, data are median of 52 patients, $n = 26$ for each group, ^a $P < 0.05$ vs proximal small bowel.

the dosage and the time of application in relation to CE. Bisacodyl is mainly activated by bacterial metabolism in the colon. Although both, sodium phosphate and bisacodyl increase luminal fluid in the small bowel, their main effect is documented in colon preparation^[22,23]. The dose of the sodium phosphates could be too low. Niv and colleagues could show that 90 mL of sodium phosphate in combination with 2 liters of water improved CE quality^[9,10]. Those studies showing a positive effect of PEG on CE visibility also used volumes of at least 2 liters^[12,15]. Therefore, not only the pharmacological effect may be responsible, but also the volume itself. Patients in our study were allowed to drink until 2 h before the examination, but no minimum volume was recommended. The volume was not documented, it was therefore not possible to examine if the individual amount of fluid intake had any effect on CE quality.

Double balloon enteroscopy (DBE) is another recent tool for examination of the small bowel^[24-26]. Requirement for more manpower and a slightly increased complication rate are some disadvantages of DBE compared to CE^[27,28]. However, DBE has a working channel which enables the examiner for example to take biopsies or intervene in bleedings^[29]. In contrast to CE no other preparation than fasting is essential when DBE is performed orally, since the small bowel content can be cleaned during the examination by means of the suction channel^[27,28].

CE is an expensive and also time consuming examination. An effective preparation is essential to minimize false results at the best possible rate. Therefore, additional studies are necessary to identify sufficient preparation procedures and to determine the impact of the volume of the preparation solution. This has to be performed in consideration of the patient's compliance, which might be reduced by the taste of the preparation solution^[30].

COMMENTS

Background

Capsule endoscopy (CE), a well accepted tool for evaluation of small bowel pathologies, has some limitations due to reduced visibility by air and residual material especially in the distal small bowel.

Research frontiers

Several forms of preparation (e.g. prokinetic drugs, laxatives and defoaming agents) have been tried to improve the quality of the examination. However, since the data is scanty and partially inconsistent, to date no standardized protocol has been recommended for bowel preparation for CE.

Innovations and breakthroughs

The effect of Prepacol® (Guerbet GmbH, Sulzbach, Germany), a combination of a saline (sodium phosphate) and a stimulant laxative (bisacodyl), on the quality and transit time of CE was tested. It has been shown that preparation with Prepacol® accelerated small bowel transit time, but had no effect on the quality of CE.

Applications

Since Prepacol® has no beneficial effect on CE the modality of preparation and the ideal time of application remains unclear.

Peer review

Interesting effort to do proper research on SB-preparation.

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Effect of Prometheus liver assist system on systemic hemodynamics in patients with cirrhosis: A randomized controlled study

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Abstract

AIM: To evaluate treatment safety and hemodynamic changes during a single 6-h treatment with the Prometheus™ liver assist system in a randomized, controlled study.

METHODS: Twenty-four patients were randomized to either the study group or to one of two control groups: Fractionated Plasma Separation Adsorption and Dialysis, Prometheus™ system (Study group; $n = 8$); Molecular Adsorbent Recirculation System (MARS)™ (Control group 1, $n = 8$); or hemodialysis (Control group 2; $n = 8$). All patients included in the study had decompensated cirrhosis at the time of the inclusion into the study. Circulatory changes were monitored with a Swan-Ganz catheter and bilirubin and creatinine were monitored as measures of protein-bound and water-soluble toxins.

RESULTS: Systemic hemodynamics did not differ between treatment and control groups apart from an increase in arterial pressure in the MARS group ($P = 0.008$). No adverse effects were observed in any of the groups. Creatinine levels significantly decreased in the MARS group ($P = 0.03$) and hemodialysis group ($P = 0.04$). Platelet count decreased in the Prometheus group ($P = 0.04$).

CONCLUSION: Extra-corporal liver support with Prometheus is proven to be safe in patients with end-stage liver disease but does not exert the beneficial effects on arterial pressure as seen in the MARS group.

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Key words: Extra-corporal liver therapy; Prometheus; Molecular Adsorbent Recirculation System; Systemic hemodynamics

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INTRODUCTION

The fact that not all patients with end-stage liver disease are suitable for liver transplantation, and the shortage of grafts enhance the need for supportive liver therapy either to secure time to stabilize hepatic functions or to enable bridging to liver transplantation. Indeed, extra-corporal liver support has turned out to be a valuable supplement to standard medical therapy (SMT)^[1]. Especially, albumin dialysis improves not only the general condition, but also both cardiovascular and renal function^[2-7] as well as the degree of hepatic encephalopathy^[1,8,9]. The Fractionated Plasma Separation, Adsorption and Dialysis (Prometheus™) system and the Molecular Adsorbent Recirculation System (MARS™) both represent such treatment modalities.

End-stage liver disease is often accompanied by a hyper-dynamic systemic circulation^[10-13]. This circulatory

change is caused by a variety of vasoactive factors, such as cytokines, prostacyclins, and nitric oxide^[14-18]. Infection or bleeding, which are frequent complications in cirrhotic patients, increase the nitric oxide production and result in aggravation of the hyperdynamic circulation^[19]. Studies comparing the hemodynamic alterations in stable patients with cirrhosis during extra-corporal intervention remain scarce though such knowledge might evidently improve patient safety and perhaps lessen reluctance towards the use of extra-corporal liver support at an early time in the treatment.

This randomized, controlled study was designed to clarify the hemodynamic effects of intervention with PrometheusTM, using MARSTM and hemodialysis as control groups, in patients with end-stage liver disease.

MATERIALS AND METHODS

Subjects

The clinical and paraclinical characteristics of the patients included in the study are listed in Table 1. The study was approved by the Ethics Committee of Copenhagen (jr. nr. KF 01-186/04) and the study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki. Twenty-six patients (10 females and 16 males) were screened and 24 (9 females and 15 males) were enrolled in the study after obtaining written and oral consent from the patient or next-of-kin. All patients included in the study were under evaluation for liver transplantation and had a right-sided heart catheterization by a Swan-Ganz catheter. Former studies from our department have evaluated the effects of a 6-h albumin dialysis on patients with acute-on-chronic liver failure as well as patients with acute liver failure^[20,21]. Consequently, the present study was designed accordingly with a 6-h treatment to match and complete the former studies. Patients were divided into three groups each receiving a 6-h extra-corporal treatment: Prometheus treatment with MARS and hemodialysis as control groups. The randomization was performed using sealed, opaque envelopes containing a computerized sequence code. Envelopes were drawn by a third person not involved in the study.

Inclusion criteria were pre-existing liver disease with decompensated cirrhosis (verified by histological examination and/or CT/MRI scanning), ascites and a history of hepatic encephalopathy or repeated variceal bleeding.

Exclusion criteria were uncontrolled systemic or intracranial bleeding, uncontrolled systemic infection, extra-hepatic cholestasis, necrotic pancreatitis, cardiovascular failure necessitating > 0.05 µg/kg per minute of norepinephrine, and a history of albumin dialysis within the last 7 d before entering the study.

Treatment

All patients were treated in our liver ward but they were admitted to our liver intensive care unit for the duration of the extra-corporal treatment. Red blood cells, platelets and fresh frozen plasma were transfused according to the attending physician's orders. Pre-treatment albumin levels were not corrected (Table 1). Measurements of pulmonary

artery core temperature, cardiac output (CO), pulmonary artery mean pressure (PAPM), pulmonary artery wedged pressure (PAWP) and central venous pressure (CVP) were obtained through thermo dilution technique using a four-lumen balloon-tipped catheter (Swan-Ganz; Baxter, Copenhagen, Denmark). Using ultrasound supervision, all patients were equipped with a double-lumen catheter in the femoral vein (24 cm, med. COMP, Harlysville PA, USA) as well as a Swan-Ganz catheter in the right internal jugular vein.

Extra-corporal treatment: It was performed using the 4008 hemodialysis machine (Fresenius Medical Care Denmark A/S, Albertslund, Denmark) capable of performing both conventional hemodialysis as well as Prometheus treatments. The 4008 machine was also combined with a MARS monitor to perform MARS treatments. The thermostat of the machine was set to 36.5°C to avoid cooling of the patients. Patients in all three groups were treated with identical blood and dialysate flow rates (225 mL/min and 500 mL/min, respectively).

Anticoagulation: Citrate/calcium-anticoagulation was used for all patients using the 4008 hemodialysis machine's built-in citrate/calcium algorithm. Calcium is automatically infused with a rate of 3.33 mmol citrate per litre of perfused blood. The citrate dose is calculated from the ionized calcium content in the patient's venous blood. Venous blood samples were taken every 30 min and immediately analyzed in heparinized syringes (Radiometer, Copenhagen, Denmark). Blood tests before and after the treatment included creatinine, platelet count and bilirubin.

The Prometheus system removes albumin bound toxins in the patient's blood by combining fractionated plasma separation and adsorption (FPSA) with conventional dialysis. The patient's blood first passes through a plasma separator with a pore size of 250 kDa. The filtered plasma fraction then passes over two adsorption columns, a neutral resin and an anion exchange resin adsorber, before it is filtered back to the systemic circulation. The blood then passes through a high-flux dialyzer (F60S, Fresenius, Denmark). The flow rate in the plasma circuit was set to 300 mL/min according to the manufacturer's recommendations.

MARS is an extra-corporal high-flux hemofiltration that removes albumin-bound toxins from the blood over a specialized hybrid (albumin-impermeable) membrane into an albumin-enriched dialysate (500 mL of 200 g/L albumin). MARS combines a standard dialysis machine with a closed-loop albumin circuit, which is re-circulated by the MARS monitor (Gambro, Lyon, France). The albumin dialysate is passed through a conventional dialyzer and afterwards a charcoal and an anion-exchanger column. The flow rate in the closed albumin circuit was set to the maximum rate of 250 mL/min.

Hemodialysis was performed using a high-flux dialyzer (F60S, Fresenius, Denmark).

Measurements

All measurements determined heart rate (HR), systolic/diastolic and mean arterial blood pressure (MAP), stroke

Table 1 Pre-treatment clinical status of patients with liver failure

No	Randomization	Sex	Age (yr)	Cause	MELD score	Child-Pugh score	Coma grade	INR	Bilirubin ($\mu\text{mol/L}$)	Albumin (g/L)	Creatinine ($\mu\text{mol/L}$)	Hg (g/L)	Platelet count ($\times 10^4/\text{L}$)	6-mo outcome
1	Hemodialysis	M	51	Alcoholic cirrhosis										
2	Prometheus	M	66	Hemochromatosis	24	C/13	II	2.5	7.1	23.6	0.7	6.2	105	Died
3	Prometheus	M	60	Alcoholic cirrhosis	22	B/9	I	1.5	2.8	39.8	2.1	5.2	126	OLT/survived
4	MARS	M	59	Alcoholic cirrhosis	25	C/14	I	2.7	8.0	25.6	0.6	5.4	22	Died
5	MARS	F	44	Alcoholic cirrhosis	24	C/13	I	2.2	11.9	17.8	0.8	6.0	148	Survived
6	Hemodialysis	F	54	Alcoholic cirrhosis	32	C/14	I	2.7	41.0	19.3	0.7	6.5	66	Died
7	Hemodialysis	F	57	PBC										
8	Prometheus	M	39	Porphyria	22	C/10	0	1.4	22.8	31.8	0.4	8.0	70	OLT/survived
9	Prometheus	F	25	Alcoholic cirrhosis	31	C/12	0	2.3	26.9	20.0	1.3	6.2	109	OLT/survived
10	Prometheus	F	59	Autoimmune hepatitis	35	C/12	I	1.8	15.6	34.0	3.3	6.2	71	Died
11	Prometheus	F	49	Alcoholic cirrhosis	25	C/12	I	2.4	9.6	29.2	0.7	7.1	63	Survived
12	MARS	F	43	Alcoholic cirrhosis	29	C/12	I	1.8	35.8	25.0	1.3	6.3	123	Survived
13	MARS	F	45	Cholest. stor. dis.	22	C/11	I	2.2	5.0	29.3	1.1	5.2	57	OLT/survived
14	MARS	M	59	Autoimmune hepatitis	26	C/10	0	1.7	35.1	31.3	0.9	8.8	31	Survived
15	MARS	M	63	Alcoholic cirrhosis	11	B/8	0	1.2	1.9	31.7	0.8	7.0	65	Survived
16	Hemodialysis	M	54	Hepatitis C	20	C/10	I	1.9	1.1	35.2	1.8	7.5	87	Survived
17	Hemodialysis	M	67	Alcoholic cirrhosis	12	B/9	I	1.4	1.6	30.4	0.7	6.6	91	OLT/survived
18	Hemodialysis	M	61	Alcoholic cirrhosis	12	B/8	I	1.2	1.2	35.1	1.4	8.3	140	Died
19	Prometheus	F	63	Unknown	10	B/8	I	1.3	1.1	40.8	0.8	8.0	146	Survived
20	Prometheus	M	57	Alcoholic cirrhosis	48	C/13	II	6.0	36.6	28.5	2.3	6.0	74	Died
21	MARS	M	65	Alcoholic cirrhosis	18	B/7	0	1.3	1.6	39.0	2.0	7.2	99	Survived
22	MARS	M	57	Hepatitis C	38	C/12	I	2.2	36.8	32.1	2.5	5.8	37	Died
23	Hemodialysis	M	59	Alcoholic cirrhosis	23	C/14	I	2.4	5.5	24.6	0.4	5.6	46	Died
24	Hemodialysis	M	52	Alcoholic cirrhosis	23	C/10	I	1.5	4.0	41.8	0.5	6.8	221	Survived

PBC: Primary biliary cirrhosis; Cholest. stor. dis: Cholesterol ester storage disease.

volume (SV), CO, CVP, PAMP, and PAWP. Baseline measurements were performed 30 min before starting the extra-corporal treatment. Throughout the 6-h treatment, hemodynamic measurements were performed and registered approximately every hour. Blood samples before and after the treatment included platelet count, international normalized ratio (INR), bilirubin, creatinine and alanine transaminase (ALT). During the treatment, we monitored the venous levels of ionized calcium as well as potassium and sodium, hemoglobin, glucose and magnesium. If necessary, substitution by continuous infusion was performed.

Calculations were performed as follows: Cardiac index (CI) ($\text{L}/\text{min}\cdot\text{m}^2$) = CO divided by the body surface area. SV (mL/beat) = CO/HR; SVRI ($\text{DS}/\text{m}^2\cdot\text{cm}^5$) = $80 \times (\text{MAP} - \text{CVP})/\text{CI}$; PVRI ($\text{DS}/\text{m}^2\cdot\text{cm}^5$) = $80 \times (\text{PAMP} - \text{PAWP})/\text{CI}$. Unless stated differently, data were expressed as mean \pm SEM.

Statistical analysis

Comparison within a group was performed using the paired *t*-test or Wilcoxon's rank sum test. For comparison between groups, the one-way-ANOVA or Kruskal-Wallis rank sum test were applied. *P* values < 0.05 were considered statistically significant.

RESULTS

Of the 24 patients included, 22 patients completed the 6-h treatment without complications. Patient number 1 and 7, both randomized to hemodialysis treatment, dropped out of the study due to repeated clotting of the dialysis filters. During the treatment, we recorded no serious adverse

events, i.e. no drop in MAP and no hemolysis or bleeding requiring therapeutic intervention. Patient number 15 from the MARS group experienced bleeding after removal of the dialysis catheter 10 h after termination of the treatment. None of the conscious patients complained of any discomfort that could be related to the extra-corporal treatment. Nine patients were discharged 2 to 19 d after the treatment; 5 patients underwent orthotopic liver transplantation (OLT) and were alive 6 mo after the transplantation, whereas 11 patients died within 4 to 143 d after participating in the study (Table 1). There was no statistical correlation between the study group and the 6-mo outcome (*P* = 0.397).

All hemodynamic measurements are listed in Table 2. A one-way analysis of variance between the three groups comparing baseline values of age, MELD score, INR, bilirubin, albumin, creatinine, hemoglobin, and platelets showed no significant differences. Pre-treatment MAP values were within the normal range (mean 76 mmHg; range 57-98 mmHg), while the other hemodynamic parameters were in accordance with the normal findings in end-stage liver disease: CO was elevated (mean 8.9 L/min; range 4.4-13.9 L/min), CI high (mean 4.6 L/min \cdot m 2 ; range 2.3-7.1 L/min \cdot m 2), and SVRI low (mean 1231 Ds \cdot s/cm $^5\cdot$ m 2 ; range 139-3616).

Only small hemodynamic changes from pre- to post-treatment in all three groups were noted. However, the MARS group showed, during the treatment, a significant increase in the systolic and diastolic blood pressure of 10.5% and 15.2%, respectively. Heart rate, CI and SV remained constant.

Paraclinical values of bilirubin, creatinine, and platelet count measured before and after the treatment were as

Table 2 Hemodynamic variables (mean \pm SEM)

Variables		Hemodialysis		MARS		Prometheus	
		Pre-treatment	Post-treatment	Pre-treatment	Post-treatment	Pre-treatment	Post-treatment
CO	L/min	8.26 \pm 1.07	8.37 \pm 1.01	8.66 \pm 0.87	8.74 \pm 0.88	9.52 \pm 1.26	9.36 \pm 1.4
HR	beats/min	74.5 \pm 5.89	78.5 \pm 8.45	79.5 \pm 6.06	81.13 \pm 6.53	82.25 \pm 6.06	81.63 \pm 6.88
MAP	mmHg	75.17 \pm 6.99	76.5 \pm 5.13	68.88 \pm 3.82	78.13 \pm 4.87	74 \pm 3.52	74.38 \pm 5
PAPM	mmHg	14.17 \pm 2.98	12.83 \pm 2.23	18.5 \pm 2.25	19.88 \pm 2.09	24.88 \pm 5.71	23.25 \pm 5.32
PAWP	mmHg	7.17 \pm 1.56	5.83 \pm 1.74	11.38 \pm 1.61	13 \pm 2.1	13.63 \pm 2.24	14.63 \pm 1.86
CVPM	mmHg	6.83 \pm 2.8	3.83 \pm 1.38	9.5 \pm 2.15	8.63 \pm 1.69	13.25 \pm 1.81	12 \pm 2.05
SV	mL	107.78 \pm 11.86	112.18 \pm 10.95	106.95 \pm 8.78	107.56 \pm 7.28	116.11 \pm 13.54	110.91 \pm 13.49
CI	L/min \cdot m ²	4.12 \pm 0.5	4.26 \pm 0.49	4.71 \pm 0.56	4.71 \pm 0.51	4.9 \pm 0.56	4.9 \pm 0.67
SVRI	DS \cdot m ² \cdot cm ⁵	1454.17 \pm 478.92	1541.17 \pm 353.82	1223.38 \pm 344.76	1372.25 \pm 271.5	1073.5 \pm 126.69	1177.38 \pm 194.2
PVRI	DS \cdot m ² \cdot cm ⁵	138.5 \pm 32.22	119.67 \pm 29.05	121.5 \pm 30.65	100.05 \pm 28.13	236 \pm 141.71	210.63 \pm 133.63

CO: Cardiac output; HR: Heart rate; MAP: Mean arterial blood pressure; PAPM: Pulmonary artery pressure mean; PAWP: Pulmonary artery wedged pressure; CVPM: Central venous pressure middle; SV: Stroke volume; CI: Cardiac index; SVRI: Systemic vascular resistance index; PVRI: Pulmonary vascular resistance index.

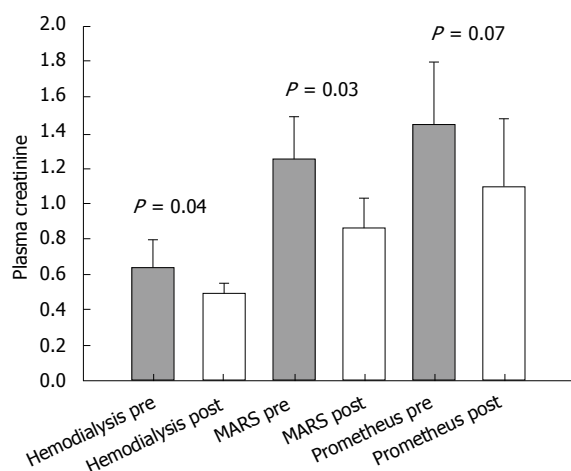


Figure 1 Plasma creatinine pre- and post-treatment using hemodialysis, MARS and Prometheus. All values are given as mean \pm SEM.

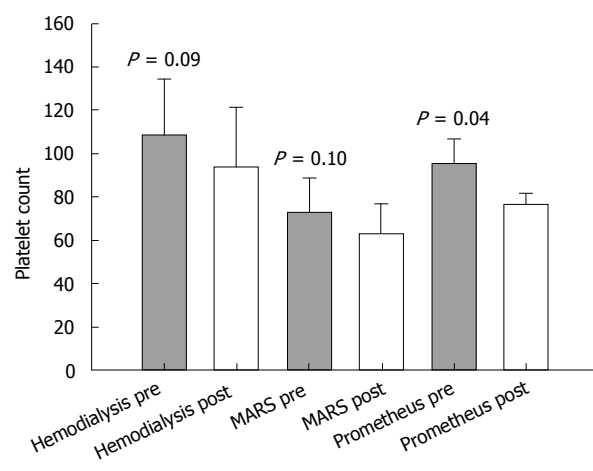


Figure 3 Platelet count pre- and post-treatment using hemodialysis, MARS and Prometheus. All values are given as mean \pm SEM.

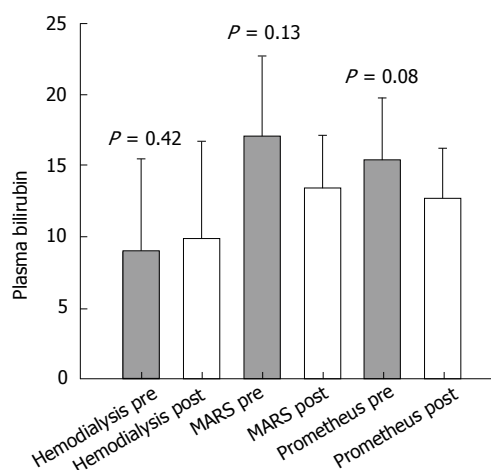


Figure 2 Plasma bilirubin pre- and post-treatment using hemodialysis, MARS and Prometheus. All values are given as mean \pm SEM.

follows: (1) Prometheus group: pre-treatment values: bilirubin (mean 15.3×10^{-2} g/L; SEM 4.4×10^{-2} g/L), creatinine (mean 1.44×10^{-2} g/L; SEM 0.35×10^{-2} g/L) and platelet count (mean 96×10^9 /L; SEM 11×10^9 /L);

post-treatment values: bilirubin (mean 12.6×10^{-2} g/L; SEM 3.6×10^{-2} g/L), creatinine (mean 1.09×10^{-2} g/L; SEM 0.39×10^{-2} g/L) and platelet count (mean 76×10^9 /L; SEM 11×10^9 /L); (2) MARS group: pre-treatment values: bilirubin (mean 17.0×10^{-2} g/L; SEM 5.6×10^{-2} g/L), creatinine (mean 1.2×10^{-2} g/L; SEM 0.24×10^{-2} g/L) and platelet count (mean 73×10^9 /L; SEM 16×10^9 /L); post-treatment values: bilirubin (mean 13.4×10^{-2} g/L; SEM 3.8×10^{-2} g/L), creatinine (mean 0.86×10^{-2} g/L; SEM 0.17×10^{-2} g/L) ($P = 0.03$), and platelet count (mean 63×10^9 /L; SEM 14×10^9 /L). (3) Hemodialysis group: pre-treatment values: bilirubin (mean 9.1×10^{-2} g/L; SEM 6.4×10^{-2} g/L), creatinine (mean 0.64×10^{-2} g/L; SEM 0.16×10^{-2} g/L) and platelet count (mean 109×10^9 /L; SEM 25×10^9 /L); post-treatment values: bilirubin (mean 9.9×10^{-2} g/L; SEM 6.8×10^{-2} g/L), creatinine (mean 0.49×10^{-2} g/L; SEM 0.06×10^{-2} g/L) and platelet count (mean 94×10^9 /L; SEM 28×10^9 /L).

The results of the paired *t*-test comparing pre- and post-treatment values in each group are shown in Figures 1-3.

Anticoagulation was performed using citrate/calcium infusion. In all patients, we followed the 4008 machine's built-in algorithm, which calculates the citrate infusion rate

from the patient's ionized calcium. Both during and after the treatment, none of the patients required calcium or citrate supplements and no correction of the pH was necessary.

DISCUSSION

In this study, we compared the systemic circulatory changes during a single treatment of Prometheus with MARS and hemodialysis as control groups in patients with end-stage liver disease. The hemodynamic differences between the three groups showed insignificant differences besides a rise in MAP in the MARS treated group. None of the patients experienced any serious adverse events. Though we have some experience both about the clinical and the hemodynamic effects of the available extra-corporal treatments, i.e. MARS and Prometheus, there is a growing recognition that patients may benefit from the initiation of extra-corporal liver support before treatment for multi-organ failure in the ICU setting is needed^[22].

We have been using both MARS and Prometheus for several years in our liver failure unit. Former studies from our unit have determined the hemodynamic changes during a single 6-h MARS treatment both in patients with acute-on-chronic liver failure (AoCLF) as well as hyper acute liver failure^[20,21]. None of the patients in this study had an acute exacerbation at the time of randomization. Thus our patients represent a group that differs from AoCLF patients but can be characterized as having a chronic liver failure with decompensation. The Child-Pugh score for our patients supports this view with 6 group B and 18 group C patients.

Arterial hypotension is a well-described adverse effect induced by hemodialysis. In the light of this fact, it is interesting that hemodynamic studies have indicated that the MARS system exerts a beneficial influence on CO, SV, SVRI^[20-23] and MAP^[20-26]. The present study cannot demonstrate such beneficial changes, neither in the Prometheus nor in the hemodialysis group; however, there was a significant increase in MAP in the MARS group. The overall lack of significant hemodynamic changes (especially on CO, SV, and SVRI as would have been expected in the MARS group) could be attributed to the fact that the patients in the present study were treated early before deterioration of their chronic liver disease: the plasma bilirubin in our patient group was 42% of the value seen in the study by Schmidt *et al.*^[20] and 48% of the mean value of all three groups in the study by Laleman *et al.*^[2].

Both the Prometheus and MARS systems are capable of removing both albumin-bound as well as water-soluble substances. As shown in Figure 2, both albumin dialysis systems, as expected, decreased the plasma bilirubin level but the removal did not reach statistical significance. This was most likely due to heterogeneity regarding pre-treatment bilirubin levels among our group of patients and a low pre-treatment bilirubin level. In addition, the absolute amount of bilirubin eliminated during a treatment depends on blood concentration: higher pre-treatment values will yield higher clearances.

The toxin concentration in the blood is generally thought to cause the hyperdynamic circulation seen in liver failure. If we consider the bilirubin level merely as an

indicator for the general level of albumin-bound toxins in the blood, it could also explain why potential positive hemodynamic effects could not be demonstrated. In our study, the pre-treatment bilirubin level was low and the toxin level would accordingly also be low. Therefore, no significant removal of bilirubin/toxins could be achieved and possible beneficial hemodynamic changes would thus become difficult to demonstrate.

No significant removal of creatinine could be demonstrated in the Prometheus group (Figure 1). This is most likely due to a low pre-treatment concentration and a high standard deviation as mentioned above. As expected, the survival of the patients was not statistically correlated to the randomized study group.

Regarding the safety of the treatment, none of the patients experienced arterial hypotension requiring cessation of the treatment. As a standard routine, the initial blood flow on the 4008 machine was set to 120 mL/min for the first 10 min and was then increased to 225 over the next 5-10 min. As mentioned, one episode of post-treatment bleeding occurred. The event was related to the removal of the dialysis catheter and occurred despite vessel compression for 15 min and a 30-min rest after dialysis-catheter removal, all according to standard guidelines.

Both the study group and the two control groups exhibited a drop in platelet count, yet, it was only statistically significant in the Prometheus group (Figure 3). Low platelet count is well known in cirrhotic patients. In addition, thrombelastography (TEG) often discloses dysfunction of the platelets. Consequently, monitoring the platelet count closely before, during, and after an extra-corporal treatment, especially when using Prometheus treatment, seems advisable as well as substitution, if necessary. Two patients in the hemodialysis group dropped out of the study due to repeated clotting. Patient number 7 showed obvious signs of hyper-coagulation: apart from clotting three hemodialysis filters, the dialysis catheter also clotted during a blood pump stop of 3-5 min. The clotting problems in the hemodialysis group opposed our former clinical experience, which had pointed at patients treated with the Prometheus system in combination with citrate anticoagulation as being most prone to clotting problems. The paraclinical data from patient number 1 and 7 did not account for the repeated clotting. Apart from the reported data, the activated prothrombin time values were within normal range for both patients (data not shown).

In conclusion, the decision to use extra-corporal liver support in the treatment of patients with end-stage liver disease hinges on many factors, with safety considerations as a major concern. The choice of treatment will depend on the risk of adverse events, and possible positive or negative hemodynamic influences between the available treatment modalities. Our study adds to the clarification of these considerations in showing that the Prometheus system does not aggravate the systemic hemodynamics; however, in our study, Prometheus does not exert an equally beneficial influence on MAP as seen during MARS treatment. We conclude that an intervention using extra-corporal liver support with albumin-dialysis should not be withheld merely because of safety considerations.

COMMENTS

Background

Patients with end-stage liver disease often need supportive liver therapy either to secure time to stabilize hepatic functions or to enable bridging to liver transplantation. Albumin dialysis improves not only the general condition, but also both cardiovascular and renal function as well as the degree of hepatic encephalopathy. Studies comparing the hemodynamic alterations in stable patients with cirrhosis during extra-corporal intervention remain scarce though such knowledge might evidently improve patient safety and perhaps lessen reluctance towards the use of extra-corporal liver support at an early time in the treatment. This randomized, controlled study was designed to clarify the hemodynamic profile of intervention with Prometheus™, using MARS™ and hemodialysis as control groups, in patients with end-stage liver disease.

Research frontiers

This paper is related to the scientific efforts to develop clinically valuable artificial liver assist devices to support patients with fulminant liver failure.

Innovations and breakthroughs

<http://www.ncbi.nlm.nih.gov/sites/entrez>. See under "liver assist devices" or Sen S, Williams R, Jalan R. Emerging indications of albumin dialysis. *Am J Gastroenterol* 2005; 100: 468-475.

Applications

The decision to use extra-corporal liver support in the treatment of patients with end-stage liver disease hinges on many factors, with safety considerations as a major concern. The choice of treatment will depend on the risk of adverse events, and possible positive or negative hemodynamic influences between the available treatment modalities. Our study adds to the clarification of these considerations in showing that the Prometheus system does not aggravate the systemic hemodynamics; however, in our study, Prometheus does not exert an equally beneficial influence on arterial pressure as seen during MARS treatment. We conclude that an intervention using extra-corporal liver support with albumin-dialysis should not be withheld merely because of safety considerations.

Terminology

Acute-on-chronic liver failure is defined as an acute deterioration in liver function in a patient with cirrhosis that results in dysfunction of other organs, such as the brain or the kidneys.

Peer review

This is an interesting and well performed study. The abstract gives a clear delineation of the research background, aim, materials and methods, results and conclusion. The design of the study is rational and reliable. The work is of the practical importance.

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RAPID COMMUNICATION

Ultrasonography in differentiation between chronic viral hepatitis and compensated early stage cirrhosis

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minate CVH from CIR. The alternative Doppler indexes can accurately differentiate chronic virus hepatitis from cirrhosis. These indexes can be used in monitoring chronic virus hepatitis and avoiding unnecessary biopsies.

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Key words: Liver cirrhosis; Virus hepatitis; Portal hypertension; Doppler ultrasonography

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Abstract

AIM: To assess the value of gray scale (GS) and colour Doppler ultrasonography (CDU) in differentiating the progression of chronic viral hepatitis (CVH) and compensated liver cirrhosis (CIR).

METHODS: Seventy-two patients and 32 normal individuals who were used as controls were studied. Forty-four patients suffered from CVH and 28 from CIR. All patients were underwent to liver biopsy. Multiple qualitative and quantitative variables were studied in liver, portal vein (PV), hepatic artery (HA) and spleen with GS and CDU. On the basis of the obtained CDU data, several known indexes were calculated. In addition, alternative indices [PV diameter (D)/time average mean velocity (V_{TAM}), HA/PV V_{TAM} ratio] were calculated and studied.

RESULTS: ROC analysis showed that PV congestion index, PV D/ V_{TAM} and HA/PV V_{TAM} indices had the best sensitivity and specificity in discriminating CVH from CIR. Stepwise discriminant analysis showed that 88.9% of the originally grouped cases could be correctly classified by the three qualitative and four quantitative variables selected as statistically significant predictors. Among the CVH patients who underwent to biopsy, statistically significant changes were found in those at fibrosis stage 5 compared to fibrosis stages 1-4.

CONCLUSION: Simple GS and CDU parameters discri-

INTRODUCTION

Chronic viral hepatitis, mainly caused by hepatitis virus B or C, results in liver parenchyma damage and inflammation and may lead to fibrosis, cirrhosis and/or hepatocellular carcinoma^[1-3]. Cirrhosis often occurs as an indolent disease and a lot of patients remain asymptomatic^[4,5] until the occurrence of decompensation, and are characterised by portal hypertension, variceal bleeding, ascites and hepatic encephalopathy.

Liver biopsy is the gold standard for diagnosis and determination of the fibrosis and necroinflammatory changes in chronic viral hepatitis and cirrhosis. However, the use of biopsy in clinical practice has some limitations related to sample errors with an estimated false negative percentage of 24% morbidity and mortality in series of blind biopsies^[6] and complications^[7].

The non-invasive assessment of chronic liver disease has been attempted by various research groups using either clinical signs^[8,9], gray scale^[10-15] and colour Doppler ultrasound (CDU) signs and indexes^[16-32], or biochemical parameters in the blood^[9]. The use of CDU in diagnosis and staging of chronic viral liver disease has been based on the hypothesis that alteration in liver haemodynamics due to chronic inflammatory changes may indirectly reflect histological alterations. Therefore, positive correlation studies have usually referred to the velocity ratio of hepatic

artery to portal vein or the resistive index in hepatic artery. However, the role of CDU remains controversial regarding the reproducibility^[33-36] and the statistical significance^[37,38] of the measurements in hepatic fibrosis and cirrhosis.

The aim of the present study was to determine the alterations in liver haemodynamics by using the Doppler indexes and measurements of spleen size that may lead to differentiation between chronic viral liver disease and compensated cirrhosis in a group of patients with a well-delineated histological profile. Furthermore, an effort was made to isolate predictive factors for discrimination between patients with chronic viral hepatitis and cirrhosis.

MATERIALS AND METHODS

Patient population

Seventy-two patients were enrolled in this controlled prospective study and divided into chronic viral hepatitis (CVH) group and cirrhotic (CIR) group. The CVH group included 44 patients (mean age 53 ± 12 years, 29 males and 18 females) with chronic viral hepatitis. Of them, 20 were positive for hepatitis B surface antigen and 24 were positive for hepatitis C serum markers. All patients underwent needle biopsy at the time of study.

The CIR group consisted of 28 patients suffering from compensated early stage cirrhosis (Child-Pugh A score, mean age 63 ± 9 years, 16 males and 13 females) due to viral hepatitis B or C. All cirrhotic patients had previous needle biopsy that confirmed their disease and endoscopic investigation of the upper GI tract.

All patients included in the study gave their written informed consent and had no known liver tumour or decompensated liver disease. The study was approved by the ethics committee of our institution.

Thirty-two healthy individuals (mean age 50 ± 15 years, 18 males and 14 females) served as controls. They were chosen from healthy volunteers with normal blood profile without evidence of liver disease. Volunteers with complex anatomy related to the hepatic artery were excluded from the study. The alcohol consumption was no more than 28 units a week (one unit = 8 g) for each individual in the control group. None of them had a history of cardiac or liver disease and risk factors for viral hepatitis, or was receiving therapy known to alter liver haemodynamics.

CDU technique and indexes

All sonographic scans were performed by a single experienced radiologist (first author), who was unaware of the clinical and laboratory data. All asymptomatic patients and healthy adults were fasted overnight before the examination. Patients did not take drugs that could affect their portal or systemic haemodynamics twenty-four hours prior to examination.

All scans were performed with the individuals lying supine using the same sonography system (ATL, HDI 3500) with a curvilinear 2.5-5 MHz transducer. The machine was supported with the proper software for direct and automatic calculation of the haemodynamic parameters based on the spectral Doppler waveform. The examination was started with the observation in gray-

scale scanning of the liver size (normal or enlarged if the midclavicular longitudinal diameter of the organ was greater than 12.6 cm ^[39], taking the value 1 for enlarged and 0 for normal), contour (nodular yes/no, taking the value 1 for yes and 0 for no) and parenchyma (homogeneity diffuse yes/no, and echogenic yes/no, taking the value 1 for yes and 0 for no). Subsequently, the examination proceeded to study CDU and a transverse section was obtained at the epigastrium to locate the proper hepatic artery in its longitudinal axis. The same method was used at the midlevel of the portal vein trunk to calculate venous indices, since no aberrant anatomy was present in the subjects participating in this study. To decrease the effect of respiration on the portal blood flow, all measurements were obtained during a short time breath-holding to avoid deep respiration. An occasional problem of overlying bowel gas was handled either by extending the scanning time or by setting a new appointment on the following day. For quantitative flow measurements, the position of the scanner was optimised until a Doppler angle of less than 60° was achieved. Haemodynamic parameters were calculated over four cardiac cycles. The sample volume size was always equal to the lumen diameter of blood vessels.

The following portal vein (PV) variables were measured: diameter (D) in cm, cross-sectional area (AR) in cm^2 , time-averaged maximum velocity (V_{MAX}) in cm/s , time-averaged mean velocity (V_{TAM}) in cm/s , blood flow volume (BF) in mL/min and the congestion index which was calculated as the ratio between cross-sectional area and time-averaged mean velocity ($\text{CI} = \text{AR}/V_{\text{TAM}}$) in cm^2/s ^[11]. The time-averaged mean portal venous velocity was determined electronically using the software package provided with the ultrasound machine.

Hepatic artery (HA) measurements included: diameter (D) in cm, cross-sectional area (AR) in cm^2 , time-averaged mean velocity (V_{TAM}) in cm/s and blood flow volume (BF) in mL/min . Resistance index (RI) of HA (percentage) was the ratio of $100 \times$ the difference of peak systolic velocity minus end diastolic velocity to peak systolic velocity, automatically given by machine's software. In addition, Doppler perfusion index (DPI)^[22] was calculated according to the formula: $\text{DPI} = \text{BF}_{\text{HA}}/(\text{BF}_{\text{HA}} + \text{BF}_{\text{PV}})$.

We also evaluated two alternative indexes for liver haemodynamics: the ratio of portal vein diameter to V_{TAM} ($\text{PV r}_1 = \text{D}/V_{\text{TAM}}$) in cm and the artery to portal vein ratio (A/P), which was calculated by the following formula: $\text{Time-averaged HA mean velocity } (V_{\text{TAM}})/\text{time-averaged PV mean velocity } (V_{\text{TAM}})$.

Two consecutive measurements of the anatomic and Doppler parameters were made in each blood vessel and the average value was taken for statistical analysis.

The spleen size was estimated by measuring the maximum craniocaudal and transverse diameters^[40,41].

Liver biopsies

Liver biopsies were fixed in formalin and embedded in paraffin. Individual histological sections were prepared and stained using standard procedures. All patients were classified on the basis of the histologic activity index according to Ishak *et al*^[42] in six fibrosis stages (F1-F6) with

the sixth (F6) to be cirrhosis stage and necroinflammatory score varying from 0 to 18 in each stage.

Statistical analysis

The quantitative variables (predictors) were compared by *t*-test and ANOVA between CVH and CIR patients and between controls. Quantitative variables between CVH patients at fibrosis stages lower than 5 and between patients with CVH at the fifth fibrosis stage were compared using Wilcoxon rank sum test since few observations were carried out in the second group and therefore parametric assumption of the *t*-test was violated. $P < 0.05$ was considered statistically significant.

Analysis of variance between the two consecutive Doppler measurements showed a very high reproducibility.

The predictive value for each of the predictors was evaluated by the area under the receiver operating characteristic (ROC) curves. Accuracy was calculated for the best cut-off value (BCV) of the current data set, defined as the highest sum of sensitivity and specificity. Stepwise discriminant analysis was performed to predict group membership from the set of predictors by the classification functions. All statistical analyses were performed using the SPSS program (version 13).

RESULTS

According to the histological findings (Table 1), 9 CVH patients were at F1 stage with necroinflammatory score (NI) ranging from 2 to 4, 9 patients at F2 stage with NI ranging from 3 to 6, 9 patients at F3 stage with NI ranging from 3 to 8, 7 patients at F4 stage with NI ranging 5 to 8 and 10 patients at F5 stage with NI ranging 3 to 9. All the 28 cirrhotic patients were at F6 fibrosis stage. No change was observed endoscopically in 8 cirrhotic patients, while first degree varices were found in 16 patients, portal gastropathy in 3 patients, first degree varices and portal gastropathy in 1 patient, respectively.

Ultrasonography

CVH group vs control group: There was a significant decrease in the portal vein mean value related to time average maximum velocity (V_{TAM}) and diameter (D) to V_{TAM} ratio between CVH and control groups ($P = 0.03$ and 0.037 , respectively). In addition, there was a significant increase in the spleen volume between the two groups ($P = 0.011$). The other haemodynamic parameters and indexes did not show any statistical significance (Table 2).

CIR group vs CVH group: There was a significant decrease related to the mean value of portal vein blood flow velocities (V_{MAX} and V_{TAM}) and blood flow (BF) between the CIR and CVH groups ($P < 0.00007$, $P < 0.00002$, $P < 0.005$ respectively, Table 1). In addition, there was a significant increase in BF of the hepatic artery and the spleen volume ($P < 0.013$, $P > 0.002$, Table 2). According to the qualitative data, liver in early stage cirrhotic patients had nodular surface, diffuse parenchymal echogenicity and was larger than that in CVH patients (Table 1).

Descriptive statistics and comparative data on qualitative,

Table 1 Liver qualitative parameters in controls, CVH and CIR groups

Variables		Chronic viral hepatitis group (CVH)	Cirrhosis group (CIR)
		<i>n</i>	<i>n</i>
Size	Enlarged	10	12
Contour	Nodular	6	13
Echogenic	Raised	11	1
Homogeneity	Diffuse	11	18

quantitative anatomic and haemodynamic variables as well as the calculated indexes in portal vein, hepatic artery and spleen are presented in Table 2. The *P* values for the quantitative variables between the two groups are listed in Table 2.

In comparison with the CVH patients, the mean values of portal vein congestion index, diameter to time average mean velocity ratio, Doppler perfusion index (DPI) as well as hepatic artery RI and HA/PV time average mean velocity ratio were all significantly increased in the early-stage cirrhotic patients ($P < 0.01$, Table 2).

CVH patients at F5 stage vs other fibrosis stages (F1-F4): Wilcoxon's test between CVH patients at F5 stage to F1-F4 showed a significant increase in portal vein congestion index (CI) ($P = 0.041$). In addition, there was a marginally significant increase in diameter, cross sectional area ($P = 0.051$) and D/V_{TAM} ratio ($P = 0.055$) in CVH patients (Table 3). Portal Vein V_{MAX} and V_{TAM} values were marginally decreased ($P = 0.056$ and 0.08 respectively) while all other variables were not significantly different (Table 3).

CIR group and F5 CVH group vs other CVH groups (F1-F4): A significant increase in portal vein's diameter ($P < 0.05$) and cross section area ($P < 0.05$) was observed, while PV V_{MAX} and PV V_{TAM} were significantly decreased ($P < 0.001$) in cirrhotic patients and CVH patients (incomplete cirrhosis) at F1-F4. In addition, a significant increase was observed in PV CI and PV D/V_{TAM} ratio ($P < 0.01$) as well as in DPI ($P < 0.05$) and HA/PV V_{TAM} ratio between the two groups of patients ($P < 0.001$) (Table 2). Spleen volume was also increased ($P < 0.05$, Table 3).

ROC and stepwise discriminant analysis of CIR group vs CVH group: Portal vein D/V_{TAM} ratio, CI and HA/PV V_{TAM} ratio had the same sensitivity of 85.71% and specificities of 59.09%-68.18% respectively, between CIR and CVH patients. Doppler perfusion index (best cut-off value of 0.29) had a very good specificity (90.91%) but a low sensitivity (42.86%) between the two groups. The area under the curve (AUC), comparing CVH group with CIR group, for each variable as measured by receiver operating characteristic curve (ROC) analysis is presented in Table 4. PV CI, D/V_{TAM} ratio and HA/PV V_{TAM} ratio under AUC curves are shown in Figure 1 A-C. The best cut-off value (BCV) for each statistically significant quantitative variable defined as the highest sum of sensitivity and specificity is summarized in Table 5.

Table 2 Quantitative and haemodynamic parameters in controls, CVH and CIR groups

Quantitative variables	Controls (a)	CVH (b)	CIR (c)	t-test		ANOVA a/b/c
				a vs b	b vs c	
Portal vein	mean ± SD	mean ± SD	mean ± SD		P value	
D (cm)	1.14 ± 0.12	1.14 ± 0.17	1.17 ± 0.19	0.88	0.481	0.739
AR (cm ²)	1.02 ± 0.20	1.03 ± 0.32	1.11 ± 0.37	0.81	0.37	0.509
V _{MAX} (cm/s)	41.56 ± 9.30	36.27 ± 9.40	27.60 ± 6.75	0.03	7.00E-05	4.00E-07
V _{TAM} (cm/s)	22.88 ± 5.69	20.64 ± 5.92	14.80 ± 3.82	0.14	2.00E-05	6.00E-07
BF	1369.76 ± 349.41	1238.06 ± 392.03	980.06 ± 319.58	0.17	0.005	6.00E-04
Hepatic artery						
AR (cm ²)	0.16 ± 0.05	0.16 ± 0.05	0.17 ± 0.06	0.75	0.772	0.867
V _{TAM} (cm/s)	29.62 ± 10.55	29.39 ± 12.70	34.06 ± 12.34	0.92	0.128	0.244
BF (mL/min)	281.07 ± 125.79	288.79 ± 163.30	341.27 ± 144.16	0.84	0.013	0.067
Indexes						
PV D/V _{TAM} [cm/(cm*s)]	0.05 ± 0.02	0.06 ± 0.02	0.09 ± 0.05	0.166	0.002	2.00E-04
PV CI (cm*s)	0.05 ± 0.02	0.06 ± 0.03	0.09 ± 0.07	0.251	0.01	0.003
HA RI	0.73 ± 0.08	0.70 ± 0.07	0.74 ± 0.04	0.252	0.169	0.257
Total BF (mL/min)	1657.21 ± 379.72	1526.85 ± 477.80	1321.33 ± 345.78	0.254	0.053	0.016
DPI	0.17 ± 0.07	0.19 ± 0.07	0.26 ± 0.11	0.41	0.001	3.00E-04
HA/PV V _{TAM}	1.34 ± 0.50	1.49 ± 0.66	2.50 ± 1.25	0.34	3.00E-05	1.00E-06
Spleen volume (cm ³)	364.63 ± 114.01	587.09 ± 408.30	937.13 ± 525.98	0.011	0.002	6.00E-06

PV: Portal vein; HA: Hepatic artery; n: Number of patients; D: Diameter; AR: Area; V_{MAX}: Time averaged maximum velocity; V_{TAM}: Time averaged mean velocity; RI: Resistance index; CI: Congestion index; BF: Blood flow volume; DPI: Doppler perfusion index; CVH: Chronic virus hepatitis; CIR: Cirrhosis.

Table 3 Quantitative variables in CVH patients at other fibrosis stages and those at fibrosis stage 5

Variables	1th-4th fibrotic stages (a)	5th fibrotic stage (b)	5th stage and cirrhotics (c)	Wilcoxon's test		t-test a vs c
				a vs b	P	
Portal vein	mean ± SD	mean ± SD	mean ± SD			P <
D (cm)	1.1 ± 0.16	1.25 ± 0.19	1.19 ± 0.19	0.051		0.05
AR (cm ²)	0.97 ± 0.28	1.25 ± 0.37	1.14 ± 0.37	0.051		0.05
V _{MAX} (cm/s)	37.5 ± 9.86	32.15 ± 6.44	28.8 ± 6.9	0.056		0.001
V _{TAM} (cm/s)	21.4 ± 6.06	18.02 ± 4.79	15.65 ± 4.27	0.08		0.001
FV (mL/min)	1227.3 ± 423.02	1274.55 ± 276.1	1057.55 ± 332.2	0.68		0.06
Hepatic Artery						
AR (cm ²)	0.16 ± 0.057	0.17 ± 0.04	0.17 ± 0.06	0.85		0.75
V _{TAM} (cm/s)	29.9 ± 13.1	27.61 ± 11.69	32.37 ± 12.35	0.60		0.41
FV (mL/min)	291.7 ± 171.32	278.79 ± 140.17	324.83 ± 143.9	0.81		0.37
Indexes						
PV CI (cm*s)	0.05 ± 0.023	0.077 ± 0.035	0.045 ± 0.02	0.04		0.01
PV D/V _{TAM} [cm/(cm*s)]	0.056 ± 0.02	0.075 ± 0.026	0.085 ± 0.046	0.055		0.01
HA RI	0.7 ± 0.07	0.697 ± 0.034	0.73 ± 0.045	0.63		0.2
TOTAL BF (mL/min)	1519.05 ± 517.6	1553.33 ± 327.8	1382.38 ± 352.3	0.8		0.2
DPI	0.19 ± 0.075	0.177 ± 0.074	0.24 ± 0.11	0.6		0.05
HA/PV V _{TAM}	1.46 ± 0.66	1.6 ± 0.67	2.26 ± 1.19	0.56		0.001
Spleen volume (cm ³)	574.06 ± 402.11	631.4 ± 448.12	856.7 ± 519	0.72		0.05

PV: Portal vein; HA: Hartery; n: Number of patients; D: Diameter; AR: Area; V_{MAX}: Time averaged maximum velocity; V_{TAM}: Time averaged mean velocity; RI: resistance index; CI: Congestion index; BF: Blood flow volume; DPI: Doppler perfusion index.

The statistically significant variables selected by the stepwise discriminant analysis are ENLARGED, ECHOGENIC, DIFFUSE, PV AR, HA RI, HA/PV V_{TAM}, and SPLEEN. The classification scores for the CVH and CIR groups are * = multiply

W₁ = -89.090 - 1.657*ENLARGED + 12.769*ECHOGENIC + 4.202*DIFFUSE - 0.764*PV AR + 235.564*HA RI + 2.961*HA/PV V_{TAM} + 0.007*SPLEEN, and W₂ = -105.029 + 0.755*ENLARGED + 10.406*ECHOGENIC + 7.683*DIFFUSE - 5.849*PV AR + 251.99*HA RI + 5.473*HA/PV V_{TAM} + 0.010*SPLEEN.

Based on these scores, we classified any new patient in the CVH group if w₁ > w₂ and in the CIR group if

w₂ > w₁. According to the classification formula, 41 CVH patients (93.18%) were correctly classified in CVH group while 23 cirrhotic patients (82.14%) were correctly classified in CIR group.

DISCUSSION

In our study, two major findings are of interest to note regarding haemodynamic parameters and indexes. The first finding is related to a statistically significant increase in PVCI between CVH patients at fibrosis stage 5 and those at fibrosis stages 1-4. At the same time, a marginally significant increase was recorded in PV diameter, cross

Table 4 Area under the curve (AUC) for each variable measured by receiver operating characteristic curve (ROC) analysis

Variables	ROC Area	Std. Err.	95% CI	P value
Liver				
Enlarged	0.60	0.06	0.49 0.71	
Echogenic ¹	0.61	0.04	0.53 0.68	
Diffuse	0.70	0.06	0.59 0.81	
Nodular	0.66	0.05	0.56 0.77	
Portal vein				
D	0.54	0.07	0.40 0.68	0.56
AR (cm ²)	0.56	0.07	0.42 0.70	0.38
V _{MAX} ¹ (cm/s)	0.77	0.06	0.66 0.88	< 0.0002
V _{TAM} ¹ (cm/s)	0.79	0.05	0.68 0.89	< 0.0001
FV ¹ (mL/min)	0.68	0.06	0.56 0.81	< 0.01
Hepatic artery				
AR (cm ²)	0.51	0.07	0.37 0.65	0.86
V _{TAM} (cm/s)	0.64	0.07	0.51 0.77	0.053
FV (mL/min)	0.63	0.07	0.49 0.76	0.07
Indexes				
PV CI (cm*s)	0.74	0.06	0.62 0.86	< 0.001
PV D/V _{TAM} [cm/(cm/s)]	0.74	0.06	0.63 0.86	< 0.001
HA RI	0.66	0.06	0.53 0.78	< 0.05
Total BF (mL/min)	0.37	0.07	0.24 0.50	0.058
DPI	0.70	0.06	0.58 0.83	< 0.005
HA/PV V _{TAM}	0.80	0.05	0.70 0.90	< 0.0001
Spleen volume (cm ³)	0.71	0.07	0.58 0.84	< 0.005

PV: Portal vein; HA: Hepatic artery; D: Diameter; AR: Area; V_{MAX}: Time averaged maximum velocity; V_{TAM}: Time averaged mean velocity; RI: Resistance index; CI: Congestion index; BF: Blood flow volume; DPI: Doppler perfusion index; CIR: Compensated liver cirrhosis; CVH: Chronic viral hepatitis. ¹Denotes CIR vs CVH.

Table 5 The best cut-of value (BCV) defined as the highest sum of sensitivity and specificity as statistically significant predictors

Variable	BCV	Sensitivity (%)	Specificity (%)
PV V _{MAX} ¹ (cm/s)	30.10	77.27	71.43
PV V _{TAM} ¹ (cm/s)	16.00	75.00	71.43
PV BF ¹ (mL/min)	1106.64	59.09	75.00
PV CI (cm*s)	0.06	85.71	65.91
PV D/V _{TAM} [cm/(cm*s)]	0.07	85.71	68.18
HA RI	0.72	71.43	54.55
DPI	0.29	42.86	90.91
HA/PV V _{TAM}	1.45	85.71	61.36
Spleen volume (cm ³)	553.00	75.00	70.45

PV: Portal vein; HA: Hepatic artery; D: Diameter; AR: Area; V_{MAX}: Time averaged maximum velocity; V_{TAM}: Time averaged mean velocity; RI: Resistance index; CI: Congestion index; BF: Blood flow volume; DPI: Doppler perfusion index. ¹Denotes CIR vs CVH.

section area and D/V_{TAM} ratio, while V_{MAX} and V_{TAM} velocities were marginally reduced. Blood flow volume in the hepatic artery and the portal vein as well as DPI index remained unchanged at all CVH fibrosis stages. Decrease in portal vein blood flow velocities related to fibrotic stage in CVH patients was firstly described by Koda *et al*^[23], who also found that portal vein blood flow volume is not significantly affected in the same patients. Tziafalia *et al*^[32] reported that portal vein blood velocities are decreased in CVH patients. In our study, PV V_{MAX} velocity was also decreased in CVH patients.

Gaiani *et al*^[25] also found that portal vein V_{TAM} velocity

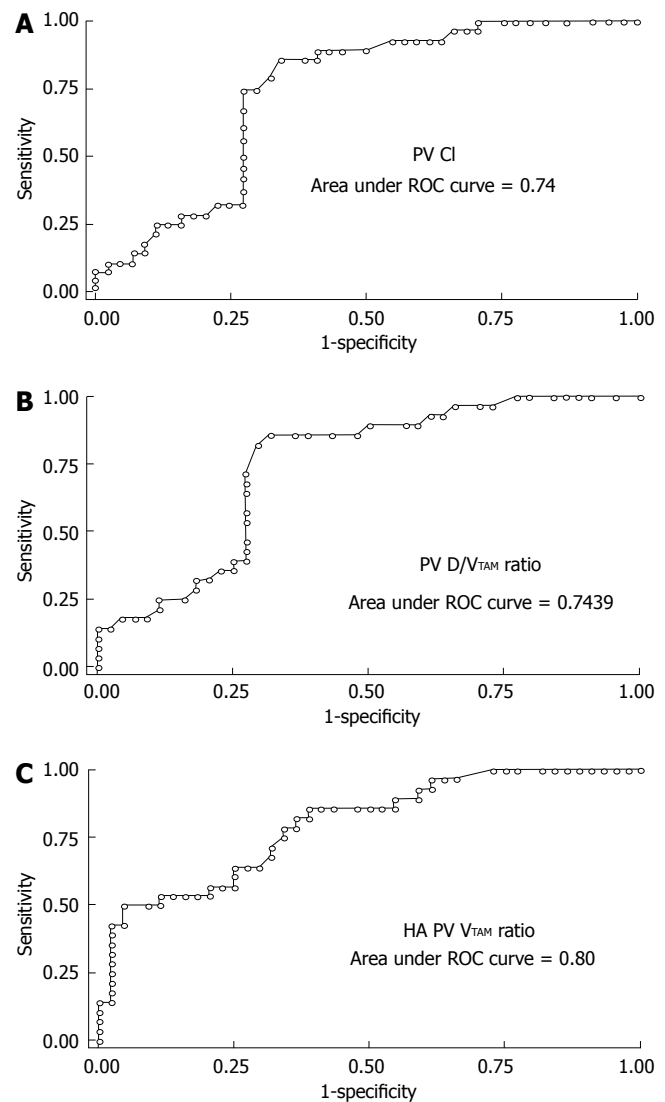


Figure 1 Portal vein congestion index (A), portal vein diameter/time averaged mean velocity (V_{TAM}) ratio (B), and hepatic arterial/portal vein time averaged mean velocity (V_{TAM}) ratio (C).

is the only haemodynamic variable that is independently associated with the histopathological diagnosis in CVH patients. Bernatik *et al*^[37] reported that V_{MAX} and V_{TAM} velocities are reduced at end-stage fibrosis, while DPI does not change significantly and progression of liver fibrosis is associated with a continuous increase in HA resistive index (RI), suggesting that Doppler parameters are not useful in assessing the stage of liver fibrosis. In our study, although the mean HA RI value was significantly increased ($P = 0.013$) in CIR group compared to CVH group, it was not affected in CVH patients at different fibrosis stages.

In our study, the portal vein diameter (D) was significantly enlarged at end-stage fibrosis patients, which is consistent with the reported data^[31]. Walsh *et al*^[29] found that hepatic artery blood flow (BF) and DPI are increased in CVH C patients at different fibrosis stages, while portal vein CI values remain unchanged. Our data do not support these findings regarding HA BF volume and DPI. However, we observed a significant change in PV CI.

The second major finding is that there were differences in anatomic, haemodynamic parameters and indexes

between CVH and CIR groups, suggesting that when early cirrhosis and portal hypertension are settled, portal vein blood flow velocity is reduced. This phenomenon is accompanied with enlargement of portal vein D and AR at end-stage fibrosis. On the other hand, hepatic artery blood flow volume increases in an effort to maintain liver blood flow volume. Portosystemic shunts and varices that may subsequently occur decrease portal hypertension. Portal vein D and AR remain for some time unchanged while blood flow velocities are further reduced. These phenomena dramatically increase most haemodynamic indexes such as portal vein CI and DPI as well as the calculated alternative ratios of $PV\ D/V_{TAM}$, $HA/PV\ V_{TAM}$.

The same observations regarding portal vein CI and DPI have been reported by other investigators^[17,20-26], which are consistent with our findings. In the present study, ROC analysis showed that portal vein CI and PVD/ V_{TAM} for the best cut-off values of 0.06 and 0.07 also had a very good sensitivity of 85.71% and a sensitivity of 65.91%-68.18% (Figures 1A and B, AUC = 0.74).

In our study, spleen volume was increased in CVH and cirrhotic patients compared with the controls, which is consistent with the findings in other studies^[19,20,22,24-26,28-31,38].

It is well known that a relative interobserver variability may limit the value for gray scale and Doppler ultrasonography^[33-36]. In this study, we tried to simplify Doppler indexes and haemodynamic parameters such as congestion index^[17] which is the ratio of area to time average mean blood velocity in portal vein. In the most recent studies, portal vein's area was assumed to be circular and is automatically calculated by the machine's software from the equation $area = \pi \cdot r^2$, because $r^2 = (diameter/2)^2$. Therefore, we replaced the area with diameter of the vessel. The $HA/PV\ V_{TAM}$ ratio at proper hepatic artery has never been described.

Stepwise discriminant analysis showed that the main predictors for discriminating CVH from CIR patients included three liver qualitative variables: ENLARGED, ECHOGENIC, DIFFUSE and four quantitative variables: PV AR, HA RI, $HA/PV\ V_{TAM}$ ratio and SPLEEN volume. By calculating the formula, 88.9% of the patients were correctly classified either in CVH group or in CIR group. The interesting finding is that the $HA/PV\ V_{TAM}$ alternative ratio was included in the classification function, while other indexes such as PVI and DPI were not included. ROC analysis confirmed the high predictive value of $HA/PV\ V_{TAM}$ index for discriminating CVH from CIR patients (best-cut-off value = 1.45, sensitivity = 85.71% and specificity = 61.36%, = 0.80, Figure 1C).

In the past, the "arteriportal index"^[43] was described as the ratio between V_{MAX} velocities in HA and PV right and left branches. We consider that the calculation of $HA/PV\ V_{TAM}$ ratio would be easier to perform. We also suggest that it can be used in routine practice since these velocities are automatically measured in most studies evaluating liver haemodynamics.

The accuracy of US in assessing diffuse liver disease has been evaluated in previous studies^[25,26]. The reported sensitivity of gray scale and US is 57%-95% in distinguishing normal from abnormal livers^[44-47]. However, attempts to identify specific pathological processes, such as fatty

infiltration and fibrosis, have produced conflicting results^[31,45], probably related to the different US criteria employed in the studies, such as distribution of the parenchymal echoes and attenuation of the ultrasonic beam. In our study, liver parenchymal changes were simply described and classified by yes or no (1 or 0). For routine practice and simplicity reasons, no further analysis was attempted in echo structure of the liver.

Percutaneous needle biopsy and histological examination of the samples are considered the gold standard for the severity of fibrosis and cirrhosis. However, several studies^[25,28] have questioned this because liver biopsies lead to false negative diagnoses of cirrhosis due to sampling errors in an estimated average of 24% pooled blind liver biopsy series^[6]. Schalm^[28] has reviewed the diagnostic methodology of liver cirrhosis and found that percutaneous liver biopsy has a sensitivity of below 85% in detection of liver cirrhosis.

It was reported that percutaneous liver biopsy sampling errors are significantly decreased when automated spring loaded true-cut needles are used^[47]. The standard of practice we used in liver biopsies is in agreement with the recently published data^[47] and unsuccessful biopsies and complications were kept at their minimum. We routinely performed a thorough sonographic investigation of the liver to assess the liver parenchyma and exclude the presence of lesions, such as a cyst. Subsequently, we performed the biopsy after marking the skin with automated cutting needles that according to our experience provide superior liver biopsy specimens in subjects with advanced fibrosis and cirrhosis; more than one samples were always taken.

On the other hand, cirrhosis is a common disease, which is frequently undiagnosed^[4,5]. The risk of biopsy (morbidity 3%, mortality 0.03%)^[7] may limit its use in screening for this disease. Finally, cirrhosis is reversible^[48], making the use of alternative non-invasive diagnostic tools essential.

Gaiani *et al*^[25] suggested that ultrasonography may be used to identify cirrhosis with a diagnostic accuracy of 80% for cirrhosis even in the absence of a typical histopathological pattern. In our study, stepwise discriminant analysis showed that its diagnostic accuracy in discriminating cirrhosis from chronic viral hepatitis patients was 82.14%.

In conclusion, gray scale and Doppler ultrasonography can accurately and non-invasively assess liver haemodynamics and discriminate CVH patients at end fibrosis stage from those at other CVH stages as well as CVH patients from early stage cirrhotic patients with compensated function. The method can easily be performed with routine upper abdominal ultrasonography, and is inexpensive and safe.

COMMENTS

Background

In recent years, many papers have been published regarding the efforts to correctly classify chronic virus hepatitis (CVH) and distinguish this entity from liver cirrhosis by bloodless means. Blood tests and ultrasonography, or other imaging modalities (CT, MRI) are used to achieve this goal. Doppler ultrasonography of portal vein and hepatic artery has gained its ground in the estimation of portal haemodynamics. Simple recording of portal vein's blood flow velocities is common in daily practice in many institutions. Haemodynamic indexes, although old as a

conception, have not been thoroughly investigated, probably due to the difficulty of producing them from the Doppler measurements in day practice.

Research frontiers

Our study investigated the value of the most popular liver haemodynamic indexes (congestion index, Doppler perfusion index, arteriportal index). We also simplified these indexes by introducing new alternative to them. Finally our data were compared to those published in literature.

Innovations and breakthroughs

The major finding of our study is that end CVH at the 5th fibrotic stage can be distinguished from those at other stages. In daily practice, incomplete and complete early stage cirrhosis may be predicted by simple haemodynamic indexes. Stepwise discriminant analysis produced a formula (including qualitative and quantitative variables) that differentiates liver cirrhosis from CVH in 80% of the originally classified cases. ROC analysis could find the best cut off values for discriminating CVH from cirrhosis. The new alternative indexes, particularly "arteriportal index" have been proved to be of great value in discriminating CVH from cirrhotic patients. The "arteriportal index" is first used in porta hepatis (portal vein's trunk and proper hepatic artery). Another major point is that the best cut-off values are similar to those from other investigators contributing to the establishment of internationally accepted values for Doppler haemodynamic indexes.

Applications

Our data suggest that the "congestion index" and alternative indexes are of value and can be added in daily practice for monitoring CVH and cirrhotic patients. Because the arteriportal index of porta hepatis is first used, more studies are needed to establish more precise best cut off values.

Terminology

"Arteriportal index": The ratio of time average mean blood velocity in proper hepatic artery to time average blood velocity in portal vein's trunk.

Peer review

In this study, the authors assessed the value of gray scale (GS) and colour Doppler ultrasonography (CDU) in differentiating the progression of CVH and compensated liver cirrhosis (CIR). Significant differences in haemodynamic parameters and indexes were found between CVH patients at fibrosis stage 5 and those at other fibrosis stages, suggesting that simple GS and CDU parameters can discriminate CVH from CIR.

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RAPID COMMUNICATION

Endoscopic band ligation and endoscopic hemoclip placement for patients with Mallory-Weiss syndrome and active bleeding

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safe for the management of active bleeding in patients with Mallory-Weiss syndrome, even in those with shock or comorbid diseases.

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Key words: Mallory-Weiss syndrome; Hemostasis; Endoscopic band ligation; Endoscopic clipping

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Abstract

AIM: To compare the hemostatic efficacy and safety of two mechanical endoscopic methods: endoscopic band ligation (EBL) and endoscopic hemoclip placement (EHP) in patients with actively bleeding Mallory-Weiss syndrome (MWS).

METHODS: A prospective randomized study to compare the efficacy and safety of EHP with EBL was performed from January 2002 to August 2005. Forty-one patients with active bleeding from MWS were treated with EHP ($n = 11$) or EBL ($n = 20$).

RESULTS: There were no significant differences between groups with respect to clinical and endoscopic characteristics. The mean number of hemoclips applied was 3.2 ± 1.5 and the mean number of bands applied was 1.2 ± 0.4 . Primary hemostasis was achieved in all patients. Recurrent bleeding was observed in one patient from the EHP group and two from the EBL group. Patients with recurrent bleeding were treated by the same modality as at randomization and secondary hemostasis was achieved in all. There were no significant differences between the two groups in total transfusion amount or duration of hospital stay. No complications or bleeding-related death resulted.

CONCLUSION: EHP and EBL are equally effective and

INTRODUCTION

Mallory-Weiss syndrome (MWS), vomiting-induced mucosal lacerations in the region of the gastroesophageal junction, is one cause of nonvariceal upper gastrointestinal (UGI) bleeding and its incidence is considered to be 5% to 15%^[1]. In most cases, MWS-related bleeding requires no intervention other than hemodynamic support^[2]. However, some patients may require intensive care^[3], especially those with risk factors such as evidence of active bleeding (for example, fresh blood hematemesis and hemodynamic instability), presence of stigmata of recurrent bleeding (such as visible vessel and adherent clots), and comorbid diseases or bleeding diathesis. Surgery or other therapeutic approaches such as balloon tamponade of the esophagus, transcatheter arterial embolization, and systemic or selective arterial infusion of vasopressin have been used to control active bleeding in patients with MWS^[4-7]. In recent decades, endoscopic treatment has been the treatment of choice^[8]. Various endoscopic techniques, mainly consisting of endoscopic coagulation or injection, have been used in the management of patients with MWS at high risk for recurrent bleeding^[9-12]. However, injection hemostasis may be incomplete for patients with a large and/or long plexuses of vessels and coagulation has the risk of producing transmural injury and perforation due to relatively thin esophageal wall^[13]. Mechanical

endoscopic methods have recently become one of the therapeutic options for treating patients with actively bleeding MWS^[13-20]. Among the mechanical methods, endoscopic band ligation (EBL) and endoscopic hemoclip placement (EHP) both has merits and problems related to the hemostatic mechanism and technical procedure itself. However, there have been few studies comparing the hemostatic efficacy of different mechanical endoscopic methods in the treatment of actively bleeding MWS. Therefore, this prospective randomized study was carried out to compare the hemostatic efficacy and safety of EBL with EHP in the treatment of actively bleeding MWS.

MATERIALS AND METHODS

Subjects

Among patients undergoing upper endoscopy at the gastrointestinal endoscopy center of Uijeongbu St Mary's Hospital from January 2002 to August 2005 because of acute UGI bleeding, all consecutive patients with endoscopically verified MWS (defined as a mucosal tear or laceration near the esophagogastric junction with active bleeding, either spurting or oozing) were considered for inclusion in the study. Written informed consent for endoscopy and participation in the study was obtained from patients or near relatives before the procedure. This research was carried out in accordance with the Helsinki declaration.

After receiving basic life support, all patients underwent endoscopy within 12 h of the onset of UGI bleeding. Patients were excluded if they were unable or unwilling to give informed consent for endoscopic therapy, if active bleeding was not present at endoscopy, or if more than one source of bleeding was identified. Patients with old adherent clots and clean-based longitudinal mucosal tears near the esophagogastric junction as the only possible origin of bleeding were treated conservatively. All patients' clinical characteristics, including age, gender, presenting symptoms, alcohol use, blood pressure, hemoglobin level, coagulopathy, and comorbid diseases, were recorded. Shock was defined as a systolic blood pressure of less than 90 mmHg and a pulse rate greater than 100 beats/min, accompanied by pallor or cold sweating.

Therapy

Forty-one patients were randomized to undergo EBL ($n = 20$) or EHP ($n = 21$) by concealed allocation determined according to a table of random numbers. All diagnostic and therapeutic endoscopic procedures were performed by members of the research team of four staff gastroenterologists. They did not participate in the postprocedure care of the patients, which was conducted by other physicians. Endoscopic procedures were performed with videoendoscopes (GIF-Q230, GIF-XQ230, Olympus Optical Co. Ltd, Tokyo, Japan). In the EBL group, an endoscopic ligating device (Pneumoactive EVL device; Sumitomo Bakelite Company, Tokyo, Japan) was used with no overtube. After endoscopic identification of the lesion, the endoscope was withdrawn and was reinserted after attachment of the band ligator. After the hood had been placed over the bleeding site, endoscopic

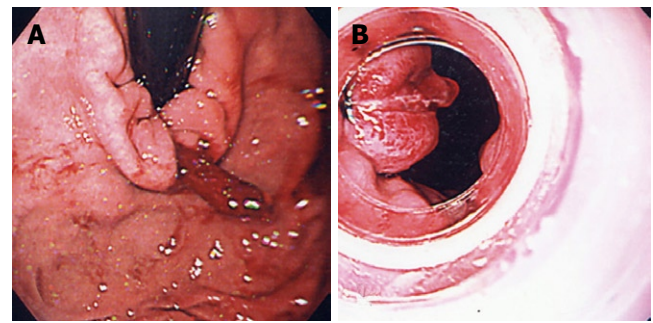


Figure 1 Endoscopic view of a Mallory-Weiss tear at the cardia of the stomach. **A:** An actively bleeding vessel; **B:** After band placement.

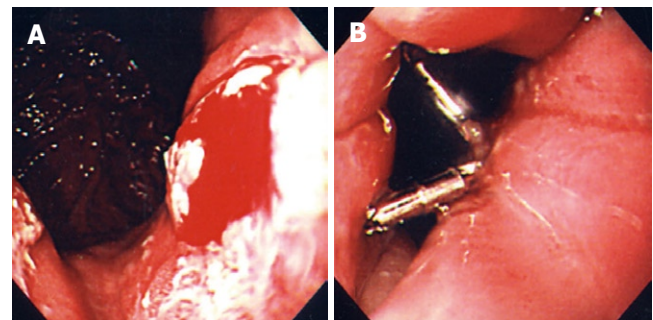


Figure 2 Endoscopic view of a Mallory-Weiss tear at the esophagogastric junction. **A:** Oozing vessel; **B:** After hemoclip application to the bleeding vessel.

suction was activated to draw the bleeding site into the banding cylinder. After suction and band release, a polypoid mass of mucosa 1 cm in diameter was formed and active bleeding stopped immediately (Figure 1). In the EHP group, hemoclips (MDS50, Olympus) were placed with a clip application device (HX-3L, HX-5 LR-1; Olympus). In terms of technique, hemoclips were applied directly on spurting or oozing vessels along with surrounding tissues (Figure 2).

Once hemostasis was achieved, the bleeding site was observed for at least 1 min. Primary hemostasis was defined as no endoscopic evidence of bleeding during this time after band ligation or after hemoclippling, during the first endoscopic session. Patients who continued to bleed, despite receiving EBL or EHP, were given endoscopic injections of epinephrine. If patients continued to bleed despite endoscopic treatment, we planned to undergo transcatheter arterial embolization or emergency surgery, depending on the status of the patient and/or the volume of blood transfused. After endoscopic treatment, all patients were observed closely. During admission, treatment included partial parenteral nutrition and intravenous administration of histamine H₂ receptor antagonists at standard doses. Evidence of recurrent bleeding included the following signs of ongoing bleeding: (1) new hematemesis; (2) fresh blood aspirated *via* a nasogastric tube; (3) continuous melena with instability of vital signs (systolic blood pressure ≤ 90 mm Hg, heart rate ≥ 100 beats/min, or orthostatic changes in systolic blood pressure of ≥ 20 mmHg or heart rate of

Table 1 Clinical characteristics and outcome of patients in endoscopic band ligation (EBL) and endoscopic hemoclip placement (EHP) groups

	EHP (<i>n</i> = 21)	EBL (<i>n</i> = 20)	<i>P</i> value
Age (yr)	49.5 ± 13.2	47.1 ± 11.9	0.58
Gender (male/female)	21/0	17/3	0.10
Alcohol use	13 (62%)	13 (65%)	1.00
Hematemesis/melena	21/1	20/2	0.61
Shock	3 (14%)	4 (20%)	0.70
Hemoglobin level (g/L)	125 ± 33	112 ± 25	0.14
Comorbid disease	9 (43%)	9 (45%)	1.00
Number of tears	1.7 ± 0.9	1.4 ± 0.6	0.29
Bleeding stigmata			0.45
Spurting vessel	3 (14%)	5 (25%)	0.63
Oozing vessel	18 (86%)	15 (75%)	
Tear location			
Distal esophagus	6 (29%)	4 (25%)	0.34
Esophagogastric junction	9 (43%)	14 (75%)	
Cardia	6 (27%)	2 (10%)	
Length of tear (cm)	2.2 ± 0.6	2.1 ± 0.5	0.34
Primary outcomes			
Primary hemostasis	21 (100%)	20 (100%)	1.00
First episode of recurrent bleeding	1 (6%)	2 (10%)	0.61
Permanent primary hemostasis	20 (94%)	18 (90%)	0.61
Secondary hemostasis	1 (100%)	2 (100%)	1.00
Other outcomes			
Blood transfusion (mean units)	2.0 ± 2.5	3.3 ± 2.9	0.14
Total procedure time (mean min)	16.7 ± 3.2	17.1 ± 2.6	0.68
Additional epinephrine injection	0	0	1.00
Bleeding-related deaths	0	0	1.00
Hospital stay (mean days)	6.7 ± 5.1	7.3 ± 3.3	0.20

≥ 20 beats/min), or a decrease in hemoglobin level of more than 20 g/L within 24 h of obtaining primary hemostasis. When recurrent bleeding was suspected, endoscopy was performed immediately and the same initial therapeutic modality used at randomization was used. After patients were discharged, the clinical outcome was evaluated by physicians who were blinded to the type of endoscopic treatment and who followed the patients for 30 d including initial hospitalization. Permanent hemostasis was defined as the absence of recurrent bleeding during this period.

To evaluate the efficacy of the two hemostatic procedures, data for each patient were collected during hospitalization and included information on demographics, medical history, presenting symptoms, initial hemodynamic status, laboratory values, rate of primary hemostasis, permanent primary hemostasis rate, number of therapeutic endoscopic sessions, need for emergency operation or transcatheter arterial embolization, bleeding-related deaths, transfusion requirements, and duration of hospitalization.

Statistical analysis

Quantitative data are summarized as the mean ± SD. The Mann-Whitney nonparametric *U* test was used to compare the mean values of continuous variables and Fisher's exact test was used for the comparison of discrete variables. *P* < 0.05 was accepted as indicating statistical significance. The analyses were performed using SPSS version 11.0 for Windows (SPSS Inc., Chicago, IL, USA).

RESULTS

During the study period, 137 patients with MWS tears underwent emergency endoscopy, but only 41 patients (30%) met the entry criteria and were randomized. Clinical and endoscopic characteristics for the patients at entry are outlined in Table 1. No differences were noted between the two groups with respect to age, gender, presenting symptoms, alcohol use, shock, hemoglobin level, coagulopathy, comorbid diseases, number of bleeding points, length of tear, stigmata of bleeding, or locations of the lacerations. Comorbid diseases in the EBL group included liver cirrhosis, coronary artery disease, stroke, and chronic renal failure; in the EHP group, comorbid diseases included liver cirrhosis, pancreatitis, valvular heart disease, and rheumatoid arthritis. However, no differences were noted between the two groups with respect to frequency of having a spurting vessel or oozing. The most common location of the tear was the esophagogastric junction in all patients (23/41, 56%; Table 1).

Primary hemostasis was obtained in all patients in each group. In the EBL group, treatment was completed in a single session (mean number of bands 1.2 ± 0.4). A single elastic band was applied in 17 patients; two bands were placed in three patients (Table 1). In the EHP group, the mean number of hemoclips applied was 3.2 ± 1.5. Two patients (10%) in the EBL group and one patient (6%) in the EHP group had recurrent bleeding; this was controlled in all three with endoscopic treatment and no patient required surgery. No differences were noted in primary outcomes including rates of primary hemostasis, recurrent bleeding, and permanent primary hemostasis. No differences were noted in the other secondary outcomes, including the number of endoscopic sessions, total procedure time, the need for additional epinephrine injection, the need for emergency operation or transcatheter arterial embolization, bleeding-related deaths, transfusion requirements, or the duration of hospitalization. No significant complications or adverse events attributable to endoscopic treatment were noted in either group, and no recurrent bleeding was noted in either group during outpatient follow-up.

DISCUSSION

MWS is a relatively common cause of nonvariceal UGI bleeding, in which an abrupt rise in abdominal pressure caused by retching or vomiting induces mucosal tears near the esophagogastric junction^[1,2]. Because most patients stop bleeding spontaneously, emergency treatment is reserved for those showing active bleeding^[3]. The rate of recurrent bleeding in patients with MWS is also lower than that for other nonvariceal bleeding^[8]. However, when bleeding is active and severe, patients require surgical treatment or nonsurgical therapeutic approaches^[4,5].

Several endoscopic methods have been used to treat actively bleeding MWS, including injection of different agents, electrocoagulation, application of hemoclips, and band ligation^[8,18]. Thermal coagulation or injection therapies have been used successfully to control active bleeding from MWS^[10-12,21]. Laine^[21] reported that

multipolar electrocoagulation significantly improved hemostasis and reduced surgery in patients with active bleeding from MWS and has been associated with few complications. However, repeated coagulation has the risk of producing transmural injury and perforation because the esophagus lacks serosa and is very thin at the tear site^[22]. Injection therapy with various agents is an effective, simple, and inexpensive first-line approach^[11,12]. Llach *et al*^[11]. Reported that endoscopic injection therapy using epinephrine and polidocanol improved outcomes (rate of recurrent bleeding, hospital stay, and transfusion requirement) compared with supportive measures alone in a prospective, randomized controlled trial. However, injection therapy can produce cardiovascular complications such as ventricular tachycardia and should be avoided in patients with a history of coronary artery disease because of the potential for systemic absorption^[23].

EBL, commonly used in variceal bleeding, has also been used to treat nonvariceal bleeding^[8]. EBL is technically easier to perform than other methods, with the lesions well viewed under direct pressure and suction from the transparent ligation cap^[20]. The use of EBL for treatment of patients with bleeding MWS has been described in several studies^[13-15,18,20,24]. Our study also demonstrated high successful rates of primary and permanent hemostasis in such cases.

EHP is acceptable for treating bleeding lesions in nonfibrotic tissues and has advantages over other hemostatic methods because it rarely causes perforation^[25]. It is effective in the management of bleeding from a Mallory-Weiss tear^[18,19,26]. Compared with cautery or sclerotherapy, it may be a safer option in the management of bleeding from MWS because of the lack of additional tissue damage with endoclips^[8,19]. In the event of a deeper extension of the tear with an esophageal perforation, the placement of endoclips can fix both problems simultaneously^[27]. EHP has been shown to be an effective alternative treatment in critically ill patients with severe gastrointestinal bleeding^[28,29]. Park *et al*^[30] reported that EHP and EBL showed similar efficacy and safety in the management of bleeding gastric Dieulafoy's lesions. However, there have been no reports comparing the efficacy and safety of EHP with those of EBL for the treatment of active bleeding from MWS.

In our study, EBL and EHP both achieved low rates of recurrent bleeding and high rates of primary hemostasis. The recurrent bleeding was successfully treated using the same method as at randomization. Shock and comorbid diseases were present in four (20%) and nine (45%) patients in the EBL group, respectively; and three (14%) and nine (43%) patients in the EHP group. Despite these adverse factors, primary hemostasis was achieved in all patients. No patient in either group required additional epinephrine injection or advanced invasive therapy such as transcatheter arterial embolization or emergency surgery, and there was no procedure-related complication in either group. Therefore, the results of our study indicate that both EBL and EHP are effective and safe for treatment of patients with actively bleeding MWS, including those with shock, comorbid diseases, and/or coagulopathy.

In conclusion, EBL and EHP are equally safe and

effective for the control of active bleeding in patients with hemodynamically unstable MWS and/or combined major diseases. Moreover, after treatment, the frequency of recurrent bleeding is low and the rate of permanent hemostasis is high.

COMMENTS

Background

Mallory-Weiss syndrome (MWS) is a relatively common cause of nonvariceal upper gastrointestinal bleeding. When bleeding is active and severe, patients require treatment. Several endoscopic methods have been used to treat actively bleeding MWS. We aimed to compare the hemostatic efficacy and safety of endoscopic band ligation (EBL) with endoscopic hemoclip placement (EHP) in the treatment of actively bleeding MWS.

Research frontiers

Endoscopic injection of epinephrine is effective treatment for MWS but has higher recurrent bleeding rate. Mechanical endoscopic methods is one of treatment options that are effective and safe for actively bleeding MWS.

Innovations and breakthroughs

EBL and EHP both have merits and problems related to the hemostatic mechanism and technical procedure itself. Although many investigators have reported the usefulness of mechanical endoscopic methods, there have been few studies comparing the hemostatic efficacy of different mechanical endoscopic methods in the treatment of actively bleeding MWS. We showed that both EHP and EBL are effective for MWS. These two methods are also used effectively in patients with hemodynamically unstable MWS or combined diseases.

Applications

This finding provides evidence that EBL or EHP in the management of actively bleeding MWS can be selected depending on physician's expertise.

Peer review

This is a report designed to compare the efficacy of two different mechanical endoscopic methods in the management of actively bleeding MWS. It is concluded that EBL and EHP are equally safe and effective. After treatment with both, the frequency of recurrent bleeding is low and the rate of permanent hemostasis is high.

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Effects of honey as a scolical agent on the hepatobiliary system

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that 10% diluted honey could be used as scolical agent safely in the presence of biliary-cystic communication.

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Abstract

AIM: To examine the effects of 10% diluted honey, which has been shown to be scolical, on the liver and biliary system and determine whether it could be used as a scolical agent in the presence of biliary-cystic communication.

METHODS: Thirty Wistar-Albino rats were divided into two groups. Honey with 10% dilution in the study group and 0.9% saline (NaCl) in the control group were injected into the common bile ducts of rats through a 3-mm duodenotomy. The animals were sacrificed 6 mo after the procedure. Histopathological, biochemical, and radiological examinations were performed for evaluation of side effects.

RESULTS: At the end of the sixth month, liver function tests were found to be normal in both groups. The tissue samples of liver and ductus choledochus of the honey group showed no histomorphologic difference from the control group. No stricture on the biliary tree was detected on the retrograde cholangiograms.

CONCLUSION: According to these results, we concluded

INTRODUCTION

Hydatid disease is still an important endemic problem in Turkey and in many other parts of the world. Dissemination of protoscolex-rich fluid during surgery is a major cause of recurrence. Instillation of scolical agent into a hepatic hydatid cyst is the most commonly employed measure to prevent this serious complication^[1]. Biliary system strictures or “caustic sclerosing cholangitis” can develop from the caustic effect of the scolical solution diffused from the cyst into the biliary system, during the surgical intervention of hydatid cysts of the liver^[2]. Surgical treatment has been used around the world for years as the primary choice of treatment. Although percutaneous treatment of hydatid cysts was considered to be contraindicated due to potential risks of anaphylactic shock and dissemination of the hydatid fluid into the abdomen, this method has been used successfully for treatment of hydatid cysts since 1980s^[3]. Up to date, many scolical agents have been used for inactivation of the cyst content, but there is no ideal agent that is both effective and safe^[4].

The surgical treatment of hydatid disease of the liver includes evacuation of the cyst with scolical irrigation and either excision or drainage of the cyst^[5]. The objectives of surgical treatment are inactivating scolices, preventing spillage of cyst contents, eliminating all viable elements of the cyst, and managing the residual cavity of the cyst.

Inactivation of scolices with various scolical agents has been tried with varying success^[6].

Honey is the foodstuff made by honeybees from the nectar of flowers or secretions from other parts of the plants, which they gather, transform together with their own specific materials, and store in honeycomb. Honey is considered as healthy and wholesome food with curative properties. It has antimicrobial effects against many bacteria and this property may be due to its osmolarity, acidity, flavonoids, aromatic acid substances, and hydrogen peroxide^[7].

In a previous study, 10% diluted honey has been shown to be highly effective on protoscolices^[8].

The aim of the present study was to investigate whether diluted honey would cause caustic sclerosing cholangitis when injected directly into the common bile duct of rats.

MATERIALS AND METHODS

Animals

Thirty Wistar-Albino female rats, weighing 225 ± 25 g, were included in this study. Animals were deprived of food 12 h before anesthesia, but had free access to water 2 h before anesthesia. No enteral or parenteral antibiotics were administered at any time. Rats were housed under constant temperature ($21^{\circ}\text{C} \pm 2^{\circ}\text{C}$) individually in wire cages with 12 h light-dark cycle. Rats that died during the study were excluded. The procedures in this experimental study were performed in accordance with the National Guidelines for the Use and Care of Laboratory Animals and approved by the Animal Ethics Committee of Ankara Research and Training Hospital.

Surgical procedure

The rats were randomly divided into two equal groups of 15 rats each. Rats were anesthetized by an intramuscular injection of ketamine HCl (Ketalar, Parke-Davis, Eczacibasi, Istanbul, Turkey; 40 mg/kg body weight) and xylazine (Rompun, Bayer, Leverkusen, Germany; 5 mg/kg body weight). All animals were allowed to breathe spontaneously during the experiments. After the abdomen was shaved and cleaned with povidone iodine, a midline laparotomy was carried out, and the intestines were covered with sterile gauze pads soaked with isotonic saline at 36°C - 38°C . A 3-mm duodenotomy was performed. Test solutions, 0.15 mL, either sterile isotonic saline solution (control group) or 10% dilutions of honey (study group) (Anzer honey, Rize, Turkey), were injected without pressure into the common bile duct with 27 gauge syringe. Immediately after the injection, the common bile duct was clamped with an atraumatic vascular clamp (bulldog). The catheter was then withdrawn. The clamp was removed 5 min later, and the duodenotomy was closed with a 6-0 polypropylene (Prolene) suture. There was no operative mortality. The study animals were kept for 6 mo, during which time they were fed with rat chow ad libitum and tap water and kept at room temperature (18°C - 20°C) in separate cages.

Blood samples were obtained 1 wk after the surgical procedure and at the end of the experimental study (6 mo

after the procedure) for liver function tests including bilirubin, aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), and gamma glutamyl transferase (GGT).

Six months after the procedure, retrograde cholangiography was performed under ketamine hydrochloride anesthesia. Midline laparotomy was carried out, and a 3-mm duodenotomy was performed. Radiopaque solution, 0.15 mL, was injected without pressure into the common bile duct using a 27 gauge syringe. Immediately after the injection, the common bile duct was clamped with an atraumatic vascular clamp and antero-posterior cholangiograms were obtained. Following cholangiography, blood samples for determination of liver function tests were obtained. Liver, common bile duct and duodenum were excised en-bloc for histopathological examination.

Biochemical and histopathological examinations

The biochemical analyses were made by an autoanalyzer (Olympus AU 640, Japan) using commercial kits.

The liver specimens of the right and left lobes and common bile duct were taken and immediately fixed in 10% neutral buffered formalin solution for one week. Tissues were washed in flowing water and dehydrated with rising concentrations of ethanol (50%, 75%, 96% and 100%). After dehydration, specimens were put into xylene to obtain transparency and were then infiltrated with and embedded in paraffin. Histological sections of the specimens in thickness of 6 μm from all the groups were stained with hematoxylin and eosin. The whole tissue blocks were sectioned and histopathological examinations were performed on systematically randomly sampled preparations by a blinded researcher. The specimens were photographed by Nikon eclipse E 600. Liver specimens were evaluated to assess the morphology of the hepatocytes, portal areas, sinusoidal lesions, cellular infiltration in the lobule or portal spaces and parenchymal lesions. Histopathological examination of the common bile duct was performed to assess the histomorphology of the epithelium, connective tissue, inflammation, fibroblastic proliferation and necrosis.

Statistical analysis

Differences between the groups were analyzed with Mann-Whitney *U* test. Statistical analysis was performed with the Statistical Package for the Social Sciences (SPSS) version 13.0 for Windows (SPSS Inc., Chicago, USA). *P* values less than 0.05 were considered to be significant.

RESULTS

Five rats from each group died within 5 d after the procedure. Two of ten died in the early postoperative period possibly due to anesthesia, and the others died because of trauma to the common bile duct and leakage into the peritoneum. The remaining 20 rats were alive until the end of the study without any problem.

Biochemical and radiological results

Liver function tests were slightly elevated 1 wk after the procedure in both groups, and this might be due to the

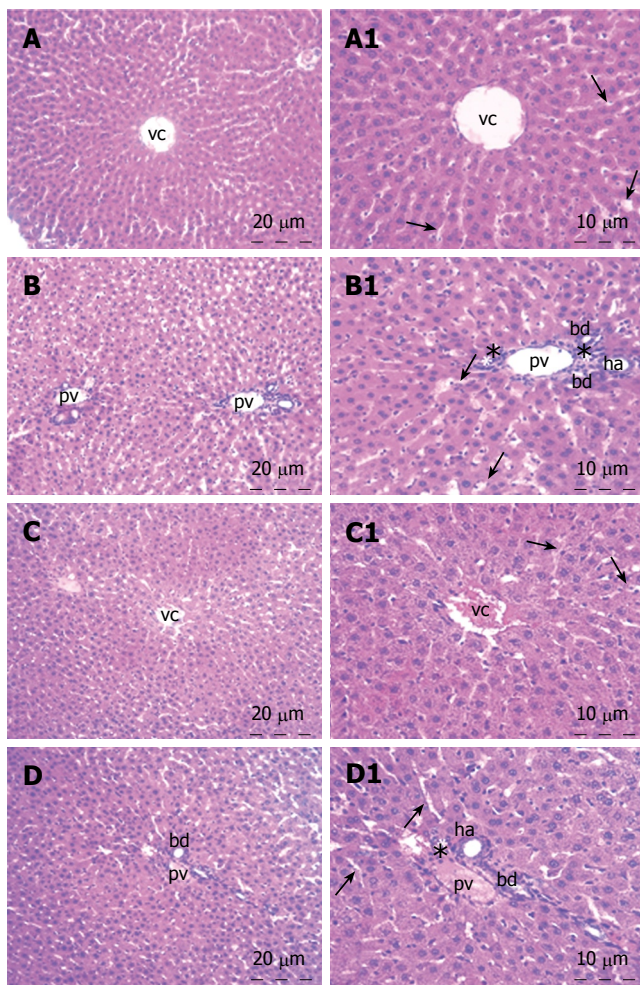


Figure 1 Liver sections stained with hematoxyline and eosin. **A, B:** Control group showing the regular architecture; **C, D:** Treatment group. vc: Central vein; pv: Portal vein; ha: Hepatic artery; bd: Bile ductules. Arrow, hepatic sinusoids; *, cell infiltration. The right column of the panel is the larger view of the left column.

cannulation and injection of common bile duct and there was no difference between the groups. At the end of the first and sixth month, liver function tests were found to be normal in both groups.

No stricture in the biliary tree was found on the retrograde cholangiograms.

Histopathological results

The histopathological evaluation of the liver sections did not reveal any difference between the control and honey groups. Polygonal shaped hepatocytes radiating outward from a central vein in the center and arranging into hepatic cords separated by adjacent sinusoids were demonstrated. Both the hepatic portal vein and hepatic artery branch and also the bile ductules in the corners of hepatic lobules were in normal architecture histologically. There was neither inflammatory cellular infiltration nor bile pigment accumulation in either group (Figure 1). When we examined the tissue samples of the common bile duct, no adverse effect of the honey was demonstrated. The epithelium of the common bile duct was penetrated into the stroma to form pits known as the sacculi of Beale. The dense connective tissue and smooth muscle were

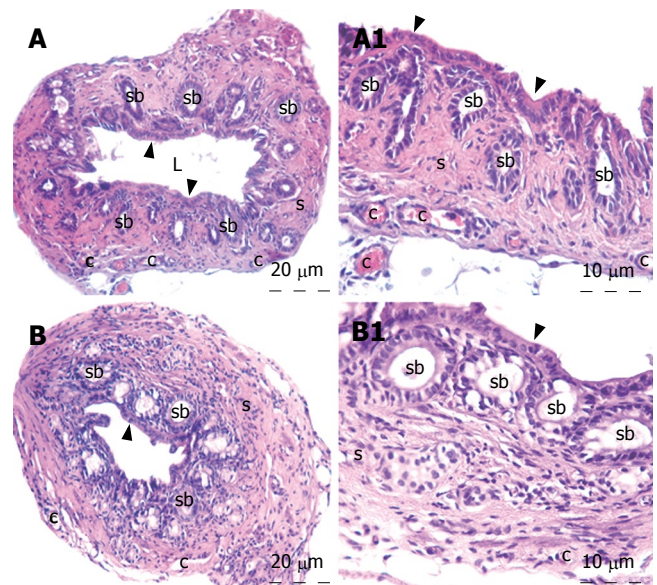


Figure 2 Common bile duct (ductus choledochus) sections stained with hematoxyline and eosin. **A:** Control group; **B:** Treatment group. L: Lumen; sb: Sacculi of Beale; c: Capillary; s: Stroma; arrow head: Simple columnar epithelium. The right column of the panel is the larger view of the left column.

in regular architecture in both groups. All three layers, mucosa, muscularis and serosa of the common bile duct were viewed normal. There was no inflammation and/or fibroblastic proliferation in the stroma of the control and honey groups. The lining cells of the sacculi of Beale were mucous gland cells that were clearly viewed in the honey group. The tissue samples of liver and common bile duct of the honey group showed no histomorphologic difference from the control group (Figure 2).

DISCUSSION

The ideal treatment for hepatic hydatid disease should completely eliminate the parasite and prevent recurrence of the disease with minimum morbidity and mortality. There are three available therapeutic modalities for hepatic hydatid disease: systemic chemotherapy, surgery, and percutaneous treatment^[9]. Meticulous packing of the operative field is necessary irrespective of the surgical technique employed with sponges soaked in scolicedal agents to inactivate the scolices which may leak from the cyst during surgical manipulation. In conventional or minimally invasive hydatid disease surgery, inactivation of the cyst content is essential, justifying the routine use of scolicedal solutions. In the presence of cystobiliary communications, the passage of these solutions may cause hepatic stasis, edema and necrosis in the hepatic tissue as well as histopathological changes in the biliary tree^[2]. Various experimental studies investigated the effects of 95% alcohol, 10% povidone iodine, 0.9%, 5.0%, 10.0%, 20.0% NaCl, 3% H₂O₂, 5% formalin, 0.5% AgNO₃, cetrimide on the liver and the biliary tree. Severe hepatobiliary complications have been reported for formalin, alcohol and 10%-20% NaCl^[10,11].

Sclerosing cholangitis may be due to immunological,

infectious, vascular, or chemical factors. In patients with hydatid disease of the liver, various factors, including injection of scolical agent into the cyst cavity, a communication between the cyst and biliary tree, and a particular sensitivity to the scolical agent seems to be necessary to promote caustic sclerosing cholangitis^[5].

Histopathological changes in sclerosing cholangitis is spotty necrosis in the liver parenchyma, widening of sinusoids, regenerative changes in hepatocytes, Kupffer cell hyperplasia, pigment accumulation, periductal fibrosis, inflammation, fibroblastic proliferation, and necrosis in the extrahepatic biliary ducts^[5,12]. In our study, no histopathological difference was detected in honey group when compared with the control group. None of the above mentioned pathological changes was present in the liver and common bile duct specimens of honey group.

The retrograde cholangiograms were all within normal limits without any evidence for biliary stricture. Liver function tests at the end of the first and sixth month were also within the normal ranges.

In a previous study, we found that honey was a potent scolical agent *in vitro*^[8]. Honey concentrations of 10% or greater killed all protoscolices. The scolical effects of honey began at the end of the third minute. Intraperitoneal application of honey resulted in adverse effects with this concentration. According to these results, we concluded that honey might be used as a potent scolical agent after the evaluation of side effects on hepatobiliary system and the *in vivo* activity. The ideal scolical agent should have rapid and complete scolical effects with minimal local and systemic side effects^[13]. No systemic side effects, such as anaphylactic reaction or hyperglycemia, and no local side effects in peritoneal surface developed with intraperitoneal administration^[8]. Since an important and life-threatening side effect of scolical agents is sclerosing cholangitis, we planned to evaluate the effects of honey on hepatobiliary system with the present study in which we did not find any side effects on hepatobiliary system evaluated by using biochemical, histological, and radiologic parameters.

In conclusion, although sclerosing cholangitis is a major complication that restrict the use of many scolical agents in the presence of a biliary-cystic communication in hydatid liver disease, our experience from the present study shows that honey can be used safely in this situation.

COMMENTS

Background

To examine the effects of 10% diluted honey, which has been shown to be scolical, on the liver and biliary system whether it could be used as a scolical agent in the presence of biliary-cystic communication.

Research frontiers

The present study investigated whether diluted honey would cause caustic sclerosing cholangitis when injected directly into the common bile duct of rats.

Innovations and breakthroughs

Sclerosing cholangitis is a major complication that restricts the use of many scolical agents in the presence of a biliary-cystic communication in hydatid liver disease, our experience from the present study shows that honey can be used safely in this situation.

Applications

Evaluates the effects of a 10% honey solution on the liver as a possible scolical agent in the treatment of hydatid cysts.

Peer review

The rationale behind this study is that the authors have previously shown that a 10% honey solution has scolical properties *in vitro* and that current scolical agents often cause sclerosing cholangitis. It is an interesting paper.

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Predictive factors for early aspiration in liver abscess

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Abstract

AIM: To determine the predictive factors for early aspiration in liver abscess.

METHODS: A retrospective analysis of all patients with liver abscess from 1995 to 2004 was performed. Abscess was diagnosed as amebic in 661 (68%) patients, pyogenic in 200 (21%), indeterminate in 73 (8%) and mixed in 32 (3%). Multiple logistic regression analysis was performed to determine predictive factors for aspiration of liver abscess.

RESULTS: A total of 966 patients, 738 (76%) male, mean age 43 ± 17 years, were evaluated: 540 patients responded to medical therapy while adjunctive percutaneous aspiration was performed in 426 patients. Predictive factors for aspiration of liver abscess were: age ≥ 55 years, size of abscess ≥ 5 cm, involvement of both lobes of the liver and duration of symptoms ≥ 7 d. Hospital stay in the aspiration group was relatively longer than in the non aspiration group. Twelve patients died in the aspiration group and this mortality was not statistically significant when compared to the non aspiration group.

CONCLUSION: Patients with advanced age, abscess size > 5 cm, both lobes of the liver involvement and duration of symptoms > 7 d were likely to undergo aspiration of the liver abscess, regardless of etiology.

INTRODUCTION

Liver abscess, particularly due to amebiasis, is an important clinical problem in tropical regions of the world and accounts for a high number of hospital admissions^[1-5]. It is usually an easily treatable condition with good clinical outcomes. There is however potential for morbidity and even mortality if proper and timely treatment is not provided^[6-9]. The standard treatment of liver abscess is the use of appropriate antibiotics and supportive care. Needle aspiration can be used as an additional mode of therapy and has been promoted by some authors for routine use in the treatment of uncomplicated liver abscess. It is suggested that needle aspiration can improve response to antibiotic treatment, reduce hospital stay and the total cost of treatment^[10-12]. Although ultrasound guided needle aspiration is fairly safe, it is nonetheless an invasive procedure requiring the passage of a wide bore needle into a highly vascular organ, and can be associated with the risk of bleeding. Needle aspirations, especially at the time of intervention has therefore remained a debatable issue and it seems important to determine its possible role in the treatment of liver abscess^[13-16].

We have used a large database of patients admitted to hospital with liver abscess in order to determine the factors associated with the likelihood of liver abscess aspiration in the treatment of patients with uncomplicated liver abscess.

MATERIALS AND METHODS

Medical records of all patients admitted to our hospital with liver abscess over a ten-year period (Jan. 1995 to Dec.

2004) were identified using the International classification of diseases 9th revision with clinical modification (ICD-9-CM-USA) and reviewed retrospectively. Patients with complicated liver abscess, generally due to rupture of abscess, were excluded from this analysis, as the indications for needle aspiration in these patients are rather different (Figure 1). Diagnosis of liver abscess was based upon clinical history and abdominal ultrasound or CT scan findings. The following data was collected in all the patients who were diagnosed with uncomplicated liver abscess: demographic information, chief complaint, duration of fever or abdominal pain, associated illnesses, malignancy and history of biliary surgery or other procedures. Results of laboratory investigations and imaging studies done at the time of admission were recorded as were the clinical course of disease, modalities of treatments used and outcome of the patients.

Patients with uncomplicated (non-ruptured) liver abscess were underwent to the following investigations: Complete blood counts, imaging by ultrasound, Indirect Hem-agglutination Assay (IHA) for amebiasis, blood culture and pus culture if the abscess was aspirated. IHA was done with serology reagent "Cellognost Amebiasis" supplied by (Dade Behring Marburg GmbH Germany) and a titer of $\geq 1:128$ was taken as diagnostic for amebic liver abscess, as per the manufacturer's recommendations. Based upon the results of these investigations, patients with liver abscess were categorized into four groups according to the following criteria: (1) Amebic liver abscess (ALA): IHA titer $\geq 1:128$ with negative blood or pus culture. (2) Pyogenic liver abscess (PLA): IHA titer $< 1:32$ with or without positive blood and/or pus culture. (3) Mixed liver abscess (MLA): IHA titer $\geq 1:128$ with positive blood and/or pus culture and (4) Indeterminate liver abscess (ILA): IHA titer between 1:32 and 1:128 with negative blood or pus culture. According to our usual practice, patients were started on standard treatment of liver abscess and if no clinical response was observed within three days, therapeutic percutaneous needle aspiration was carried out at the discretion of the treating physician. Needle aspiration was done under local anesthesia and ultrasound guidance without catheter drainage and the procedure was repeated in 3-4 d if optimal response was not obtained. Antibiotics were continued for 10-14 d for ALA, 4-6 wk for PLA and mixed infection and 2-6 weeks for indeterminate abscess.

Statistical analysis

A descriptive analysis was done for demographic, clinical and radiographic features and results were presented as mean \pm SD for quantitative variables and number (percentage) for qualitative variables. In univariate analyses, differences in proportions for the group of patient underwent to needle aspiration and no aspiration was done by using the Chi-square test or Fisher exact test where appropriate. One-way analysis of variance and independent samples *t*-test were used to assess the difference of means for contrasts of continuous variables. Multiple logistic regression analysis was done and factors associated with likelihood of abscess aspiration were identified.

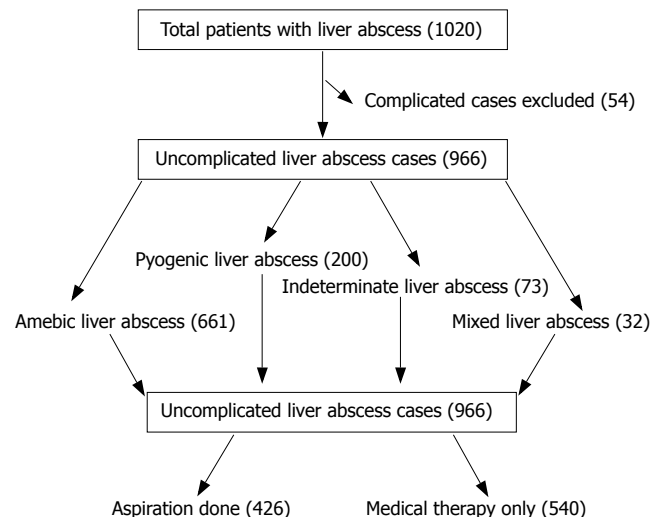


Figure 1 Flow diagram of the patients with liver abscess and treatment received.

RESULTS

A total of 1020 patients with liver abscess were admitted during the study period. Fifty four patients with complicated liver abscess were excluded from the study and 966 patients with uncomplicated liver abscess were evaluated (Figure 1). The mean age was 43 ± 17 years and 738 (76%) were males. The abscess was diagnosed as amebic (ALA) in 661 (68%), pyogenic (PLA) in 200 (21%), indeterminate (ILA) in 73 (8%) and mixed (MLA) in 32 (3%) patients. Clinical features of the patients in different types of liver abscess are presented in Table 1. Five hundred and forty patients responded to medical therapy alone; adjunctive percutaneous aspiration was performed in 426 patients. Demographic, clinical and laboratory features of the two groups are compared in Tables 2-4. There were significant differences between aspiration and non aspiration groups for many covariates.

In the aspiration group, more patients were older than 55 years (OR = 1.008; 95% CI = 1.0-1.01), duration of symptoms was more than 7 d (OR=1.60; 95% CI = 1.21-2.11), they were more likely to be jaundiced (OR = 1.55; 95% CI = 1.18-2.17), have tender hepatomegaly (OR = 0.68; 95% CI = 0.48-0.97) and hospital stay of more than 5 d (OR = 2.99; 95% CI = 1.75-2.99), as compared to the non-aspiration group (Table 2).

In the laboratory features the meaningful predictors of aspiration were elevated total bilirubin (OR = 1.09; 95% CI = 1.04-1.15), ALT (OR = 1.002; 95% CI = 1.0-1.004), alkaline phosphatase (OR = 1.002; 95% CI = 1.001-1.003), total leukocyte count (OR = 1.01; 95% CI = 1.004-1.03) and platelet count (OR = 1.001; 95% CI = 1.0-1.002), whereas relatively low serum albumin (OR = 0.59; 95% CI = 0.44-0.80) was found in the aspiration group as compared to the non aspiration group (Table 3).

The aspiration group, when compared with the non-aspiration group, was also found to have more patients with abscess sizes larger than 5 cm (OR = 1.59; 95% CI = 1.21-2.09), multiple abscesses (OR = 1.66; 95% CI = 1.23-2.24), involvement of both lobes of the liver (OR =

Table 1 Clinical features of the patients with different types of liver abscess *n* (%)

Characteristics	Amebic abscess (<i>n</i> = 661)	Pyogenic abscess (<i>n</i> = 200)	Mixed abscess (<i>n</i> = 32)	Indeterminate abscess (<i>n</i> = 73)	<i>P</i> value
Gender					
Male	568 (86)	158 (79)	27 (84)	66 (90)	0.06
Female	93 (14)	42 (21)	5 (16)	7 (10)	
Age					
< 55 yr	471 (71)	146 (73)	25 (78)	52 (71)	0.83
≥ 55 yr	190 (29)	54 (27)	7 (22)	21 (29)	
Duration of symptoms ¹					
≥ 7 d	453 (69)	136 (68)	24 (75)	47 (64)	0.752
< 7 d	208 (32)	64 (32)	8 (25)	26 (36)	
Jaundice (%)					
No	534 (81)	170 (85)	26 (81)	64 (88)	0.341
Yes	126 (19)	30 (15)	6 (19)	9 (12)	
Tender hepatomegaly					
Yes	553 (84)	179 (90)	26 (81)	57 (78)	0.085
No	107 (16)	21 (10)	6 (19)	16 (22%)	
Diabetes mellitus					
No	546 (83)	166 (83)	25 (78)	60 (82)	0.925
Yes	115 (17)	34 (17)	7 (22)	13 (18)	
Treatment					
Aspiration not done	375 (57)	109 (54)	12 (37)	44 (60)	0.151
Aspiration done	286 (43)	91 (46)	20 (63)	29 (40)	
Patient outcome					
Alive	649 (98)	192 (96)	31 (97)	72 (99)	0.352
Died	12 (2)	8 (4)	1 (3)	1 (1)	

¹Fever or abdominal pain.**Table 2** Clinical features of patients with liver abscess

Characteristics	Aspiration group (<i>n</i> = 426)	Non aspiration group (<i>n</i> = 540)	Odds ratio	95% CI	<i>P</i> value
Gender					
Male	365 (85.7)	454 (84.1)	1.13	0.79-1.61	0.490
Female	61 (14.3)	86 (15.9)	1.00		
Age					
< 55 yr	282 (66.0)	412 (76.0)	1.00	1.2-2.2	0.001
≥ 55 yr	144 (34.0) ^a	128 (24.0)	1.60		
Duration of symptoms ¹ (<i>n</i> %)					
< 7 d	111 (26.1)	195 (36.1)	1.00		
≥ 7 d	315 (73.9) ^a	345 (63.9)	1.60	1.21-2.11	0.001
Jaundice (<i>n</i> %)					
Yes	91 (21.4) ^a	80 (14.8)	1.55	1.18-2.17	0.009
No	335 (78.6)	459 (85.2)	1.00		
Diabetes mellitus (<i>n</i> %)					
Yes	70 (16.4)	99 (18.3)	0.87	0.62-1.22	0.440
No	356 (83.6)	441 (81.7)	1.00		
Patient outcome (<i>n</i> %)					
Alive	414 (97.2)	530 (98.1)	1.00	0.65-3.59	0.320
Died	12 (2.8)	10 (1.9)	1.53		
Hospital stay (<i>n</i> %)					
< 5 d	126 (29.6)	265 (49.1)	1.00		
≥ 5 d	300 (70.4) ^a	275 (50.9)	2.29	1.75-2.99	0.001

Variable compared to reference category (OR = 1), ^a*P* < 0.05; ¹Fever or abdominal pain.**Table 3** Laboratory features of the patients with liver abscess

Characteristics (mean ± SD)	Aspiration group (<i>n</i> = 426)	Non aspiration group (<i>n</i> = 540)	Odds ratio	95% CI	<i>P</i> value
Total bilirubin (mg/dL)	2.55 ± 3.4	1.86 ± 2.2	1.090	1.04-1.15	0.001
ALT (IU/L)	81.29 ± 98.87	66.18 ± 75.9	1.002	1.000-1.004	0.010
Alkaline phosphatase (IU/L)	18.45 ± 141.3	86.8 ± 117.2	1.002	0.001-1.003	0.001
Albumin (g/L)	2.24 ± 0.50	2.42 ± 0.6	0.590	0.44-0.80	0.001
Serum creatinine (mg/dL)	1.37 ± 1.20	1.25 ± 0.8	1.130	0.99-1.28	0.070
Leukocyte counts (10 ³ /mm ³)	20.09 ± 9.61	18.67 ± 8.03	1.010	1.004-1.030	0.010
Platelet counts (10 ³ /mm ³)	357.69 ± 162.40 ^a	328.33 ± 143.98	1.001	1.000-1.002	0.004

Variable compared to reference category (OR = 1), ^a*P* < 0.05; ALT: Alanine aminotransferase.**Table 4** Radiological features of patients with liver abscess (%)

Characteristics	Aspiration group (<i>n</i> = 426)	Non aspiration group (<i>n</i> = 540)	Odds ratio	95% CI	<i>P</i> value
No. of abscess					
Single abscess	303 (71.1)	434 (80.4)	1.00		
Multiple abscesses	123 (28.9) ^a	106 (19.6)	1.66	1.23-2.24	0.001
Site of lobe involvement					
Right lobe	301 (70.7)	415 (76.9)	0.97	0.67-1.40	
Left lobe	59 (13.8)	79 (14.6)	1.00	-	0.010
Both lobes	66 (15.5) ^a	46 (8.4)	1.92	1.15-3.18	
Presence of gallstones					
Yes	8 (1.9) ^a	11 (2.0)	0.92		
No	418 (98.1)	529 (98.0)	1.00	0.36-2.30	0.860
Chest radiograph					
Normal	235 (55.2)	334 (61.9)	1.00		
Abnormal ¹	191 (44.8) ^a	206 (38.1)	1.31	1.01-1.70	0.030
Size of abscess					
≤ 5 cm	121 (28.4)	209 (38.7)	1.00		
> 5 cm	305 (71.6) ^a	331 (61.3)	1.59	1.21-2.09	0.001

¹Raised right hemi diaphragm, pleural effusion, and right lung base atelectasis. Variable compared to reference category (OR = 1), ^a*P* < 0.05.

for aspiration of liver abscess were age > 55 years, (OR = 1.6; 95% CI = 1.2-2.2), size of abscess more than 5 cm (OR = 1.6, 95% CI = 1.2-2.09), both lobes of the liver involvement (OR = 2.2, 95% CI = 1.5-3.4) and duration of symptoms lasting more than seven days (OR = 1.6, 95% CI = 1.2-2.1) (Table 5).

The number of abscesses ranged from 1-6 (median 2). None of the patients with uncomplicated liver abscess required surgery. In 403 (42%) patients, only one aspiration session was done and in 23 (2%) patients, 2-3 aspiration sessions were done before full recovery.

Twelve patients died in the aspiration group, although this mortality was not statistically significant when compared with the non aspiration group (Table 2). No deaths occurred as a direct complication of the needle aspiration.

DISCUSSION

The use of needle aspiration in the treatment of uncomplicated liver abscess remains a debatable issue. Although

1.92; 95% CI = 1.15-3.18) and abnormal chest X-rays (OR = 1.31; 95% CI = 1.01-1.70) (Table 4).

Using multiple logistic regression, independent predictors

Table 5 Independent predictors for aspiration of liver abscess

Factor	Coefficient	Adjusted odds ratio	95% CI for adjusted odds ratio	Wald P-value
Age				
< 55 yr	0	1 ¹		
≥ 55 yr	0.5135	1.6	1.2-2.2	0.001
Size of abscess				
≤ 5 cm	0	1 ¹		
> 5 cm	0.5324	1.6	1.2-2.09	0.001
Location of abscess				
One lobe	0	1 ¹		
Both lobes	0.7958	2.2	1.5-3.4	0.001
Duration of symptoms				
< 7 d	0	1 ¹		
≥ 7 d	0.4542	1.6	1.2-2.1	0.001

The parameter coefficient, adjusted odds ratio, 95% CI and Wald P-value, were estimated using multiple logistic regression. ¹Reference category.

most of these patients respond to antibiotics and supportive care, a significant number eventually require needle aspiration which is generally done at a later stage, while medical therapy alone is considered as inadequate, resulting in an extended hospital stay^[17-21].

An early decision regarding aspiration of liver abscess is therefore important as it is likely to reduce the length of hospital stay and hence the cost of treatment. On the basis of patient characteristics at the time of presentation, using a large data set, we have identified some factors that are associated with aspiration of liver abscess irrespective of the underlying etiology but we were unable to evolve a model for aspiration with good power.

Most patients in this series also recovered completely on appropriate antibiotics and supportive care. However in a substantial number of patients, percutaneous needle aspiration was additionally done for complete recovery. Based upon a comparative analysis between the two groups, patients who underwent aspiration were older, had larger or multiple abscesses and longer duration of symptoms than patients who recovered completely on medical therapy alone. Underlying etiology of amebic, pyogenic, mixed or indeterminate infection was not found to be a determinant for aspiration.

This study also reflects the difficulties sometimes faced by clinicians in determining the etiology of liver abscess. In this series, 68% of the 966 patients were diagnosed to have ALA, reflecting the burden of amebic infection in tropical regions of the world^[3].

The diagnosis of ALA is usually based on clinical and radiological features along with a positive IHA Entameba titer^[1-3]. However none of these features are diagnostic and, in clinical practice, confusion can often arise as to the accurate diagnosis of ALA. For example a solitary abscess in the right lobe of the liver is considered to be highly suggestive of ALA^[3,6,22], which was true for 79% of ALA patients in this study. However a predominantly single abscess involving the right lobe was also seen in 68% of patients with PLA, suggesting that the presence of a single right lobe abscess should not exclude the diagnosis of PLA even in areas endemic for ALA. Similarly, although multiple abscesses involving both lobes were present more

commonly in patients with PLA^[23-25], they were also seen in 21% of patients with ALA.

The IHA Entameba titer is used to confirm the diagnosis of ALA. A high titer of IHA in invasive amebiasis is seen due to prolonged antigenic stimulation of absorbed liver abscess material^[2]. However some issues should be considered when amoebic IHA is used in clinical practice. There is firstly the problem inherent in any serological assay, and that is the time lag required for the test to become positive so that the first reading may not achieve diagnostic values^[2]. Secondly, in an endemic area, the appropriate cut off value for a positive test needs to be defined, keeping in mind the background positivity due to repeated (sub clinical) exposures^[1-3]. Titers of ≥ 1:128 were considered as diagnostic for ALA in this study, based upon the manufacturer's recommendations and current literature^[1,2]. However the higher the titer, the better is its diagnostic value considered.

Patients with pyogenic liver abscess (PLA) in this study has shown some differences in their clinical profile compared with other reported series; they were younger (mean age of 43 ± 17 years) and the etiology was predominantly cryptogenic^[18,19].

Although gallstones were present more frequently in PLA compared to patients with ALA, they were not associated with ascending cholangitis in the present series as reported previously^[15,16]. The reasons for these differences are not clear. However other reports also show series of patients with PLA where the primary focus of infection was not known^[26-28].

Mixed abscess in this series comprised of 32 (3%), similar to the 4%-5%. Prevalence reported in the literature^[20]. Mixed abscesses are basically ALA with secondary bacterial infection and their outcome was similar to ALA in the current series (Table 5) while in some studies mixed abscess has high mortality^[29,30].

Patients with indeterminate liver abscess behaved like ALA as regards response to treatment and outcome of disease. However the IHA titer failed to rise, even on successive testing in some cases. This may be due to a technical failure of the serological tests in these patients for various reasons, including a depressed immune status.

In conclusion, based upon a retrospective analysis of a large series of patients, we have found that if the patient with liver abscess is older in age, the abscess is more than 5 cm in size, both lobes of the liver are involved and the duration of symptoms is more than a week, then these patients are more likely to undergo percutaneous aspiration regardless of the etiology of abscess. A prospective study to validate these observations is underway, and if found accurate, it is likely to have an impact on cost effective approaches and quality of life in the management of such patients.

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COMMENTS

Background

Liver abscess, especially the amebiasis is more prevalent in the tropical region of the world due to poor hygiene and sanitation. The standard treatment of liver abscess is the use of appropriate antibiotics and supportive care. Needle aspiration can be used as an additional mode of therapy and has been promoted by some authors for routine use in the treatment of liver abscess. It is suggested that needle aspiration can improve responses to antibiotic treatment, reduce hospital stay and the total cost of treatment. Needle aspirations, especially at the time of intervention has therefore remained a debatable issue and it seems important to determine its possible role in the treatment of liver abscess.

Research frontiers

On the basis of a large dataset, we determine the factors associated with the likelihood of liver abscess aspiration in the treatment of patients with uncomplicated liver abscess.

Innovations and breakthroughs

Based upon a retrospective analysis of a large series of patients, we have found that if the patient with liver abscess is older in age, abscess is more than 5 cm in size, both lobes of the liver are involved and duration of symptoms is more than a week then these patients are more likely to undergo percutaneous aspiration regardless of the etiology of abscess.

Applications

A prospective study to validate these observations are underway, and if found accurate, it is likely to have an impact on the cost effectiveness and quality of life in the management of such patients.

Peer review

This paper by Khan *et al* reports information on predictive factors for aspiration in liver abscess. It is a retrospective study based on a very large series of cases. A number of factors showed predictive values for aspiration in liver abscess. Although retrospective, this study is well-designed and performed and the findings are sound for the clinical arena.

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RAPID COMMUNICATION

Enhancement of CD4⁺ T cell activities and modulation of Th1/Th2 lineage development in radiated tumor-bearing rats treated with male zooid of *Antheraea pernyi* extracts

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control rates were $63.08\% \pm 6.43\%$, $69.86\% \pm 7.12\%$ and $35.30\% \pm 7.67\%$, respectively. CD4⁺ T and CD8⁺ T cells in the peripheral blood of the simple radiation group were fewer than in control group. In the MZAPE and radiation plus MZAPE groups, the number of CD4⁺ T cells was higher while CD8⁺ T cells was lower than in the control and simple radiation groups. Expression of IL-2 and INF- γ in the radiation group was lower than in control group, and significantly enhanced during MZAPE therapy ($P < 0.05$). Expression of IL-4 and IL-10 in the radiation group had no significant changes compared with the control group, and decreased significantly after MZAPE treatment ($P < 0.01$).

CONCLUSION: MZAPE administration may help improve the immune function of the radiated tumor-bearing rats and reverse the radiation-induced immune inhibition by promoting the proliferation of T helper cells and inducing the transdifferentiation from Th2 to Th1.

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Key words: *Antheraea pernyi*; Male zooid; Rats; Radiotherapy; Immune suppression

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Zhao WH, Li L, Zhang B, Zhang WD, Zong M, Tang JD, Zhang HY, Li S. Enhancement of CD4⁺ T cell activities and modulation of Th1/Th2 lineage development in radiated tumor-bearing rats treated with male zooid of *Antheraea pernyi* extracts. *World J Gastroenterol* 2008; 14(13): 2094-2099 Available from: URL: <http://www.wjgnet.com/1007-9327/14/2094.asp> DOI: <http://dx.doi.org/10.3748/wjg.14.2094>

Abstract

AIM: To investigate whether supplementation of male zooid of *Antheraea pernyi* extracts (MZAPE) could enhance immune function of radiated tumor-bearing rats.

METHODS: Eighty male Wistar rats were randomly divided into a control group, a simple radiation group, a MZAPE group, and a radiation plus MZAPE group. With the tumor model established by implanting Walker-256 ascites tumor cells, tumor weight and tumor control rate were calculated. The rats in the simple radiation and radiation plus MZAPE groups were underwent to radiation at 10 Gy within 2 d. In the MZAPE and radiation plus MZAPE groups, the MZAPE was gavaged at a dose of 16.53 mg/kg once a day for 7 d. T cell subsets in peripheral blood were determined by flow cytometry and the expression of IL-2, INF- γ , IL-4 and IL-10 in sera were determined by ELISA on the 8th d.

RESULTS: The tumor weight of simple radiation group, MZAPE group and radiation plus MZAPE group was lower than that of control group ($P < 0.01$) and tumor

INTRODUCTION

Patients and experimental animals with advanced cancer often exhibit a poorly functioning immune system^[1], which was manifested by allergy to skin-test antigens^[2], decreased T-cell proliferation^[3], alterations in signal transducing molecules^[4], reduced CD4:CD8 ratios^[5] and deficient production of Th1 cytokines^[6]. These alterations correlate with the severity of the disease and poor survival^[7]. Large doses of radiotherapy in tumor treatment

is associated with normalization of cytokine producing capacity in cancer patients^[6]. Moreover it will inevitably hurt normal tissues and organs, resulting in a weak body and poor immune functions. Radiotherapy-induced immune suppression could contribute to the spread of the disease and constitute a barrier to immunotherapeutic interventions. Many experimental models have confirmed the correlation between the diversity of clinical or pathological features in immune related diseases in cancer patients. Multiple factors may contribute to radiotherapy-induced immune suppression. These include the number of immunocompetent cells, which are mainly the T lymphocytes that are most sensitive to radiation, and the balance of Th1/Th2 cytokine production^[8]. The T cell subgroups are general indicators for evaluating the immune state and immune competence of the body. The Th1/Th2 classification scheme is useful in terms of correlation between overall cytokine production patterns and clinical outcomes in a variety of pathological states^[9]. INF- γ secreted from Th1 cells is known to stimulate the differentiation of naive CD4⁺ T cells into Th1 cells and to inhibit the proliferation of Th2 cells^[10]. In addition, IL-4 and IL-10 secreted from Th2 cells are known to induce the differentiation of naive CD4⁺ T cells to Th2 cells and to inhibit the function of Th1 cells^[11,12]. In addition, the regulation of the immune balance of Th1/Th2 cell responses has been shown to be critically important for anti-tumor immune responses, such as inhibition of tumor growth and metastasis, and improvement of survival rate^[13-15]. As described above, the tumor tissue and radiotherapy-induced immune suppression mainly express Th2 cytokines, resulting in a drift from Th1 to Th2, which further induces immune suppression. Therefore, it is important to improve immune function and to drift Th2 to Th1. Therapeutic interventions aimed at protecting the immune system from damage caused by radiotherapy in cancer patients may, therefore, enhance their immune competence. Seo^[16] demonstrated that Chinese herbal medicine could change the Th1/Th2 balance by directly weakening the activities of the T cells, and the Th1 cytokines could take the predominant position.

The male zooid of *Antheraea pernyi* (MZAPE) has long been used as a pure preparation of traditional Chinese medicine to treat many illnesses and promote longevity. Our previous study demonstrated that the concentrated liquor of the male zooid could improve immune function, promote recognition and lethal effects toward tumor cells, and it possessed a unique priority for adjunctive therapy against tumors^[17]. We investigated the mechanism regarding the reversion of radiation-induced immune suppression by the active ingredients of MZAPE extracts in tumor-bearing rats.

MATERIALS AND METHODS

Materials

The unmated MZAPE was obtained from the Silkworm Research Institute of Shandong Academy of Agricultural Science. In total, 250 g of zooid was ground into powder and then passed through a mesh screen (#80) and suspended in aqueous ethanol (95%; 20 L) for 24 h. After

filtration, the residue was resuspended in aqueous ethanol (95%; 20 L) for an additional 24 h and refiltered. We then concentrated MZAPE by combined liquid-phase filtration.

Experimental animals and grouping

Eighty male Wistar rats with an average weight of 120 ± 10 g were purchased from the Animal Center of Shangdong University. All animals were housed in groups of 4-6 in 29 cm \times 18 cm \times 13 cm polyethylene cages. The animal room was maintained at 22°C-24°C on a fixed light: dark cycle (12 h: 12 h). Rats were looked after according to the Guide for the Care and Use of Laboratory Animals (National Academy Press, Washington, D.C.). Our hospital Ethical Committee for Animal Welfare approved all the experiments. Hen egg lysozyme (HEL) was purchased from Sigma-Aldrich (St. Louis, MO) and recrystallized three times. Rats were immunized via the hind footpads with 1 nmol/rat of native HEL emulsified in colonization factor antigen (CFA) (Difco, Detroit, MI). The rats were weighed and randomly divided into a control group, a simple radiation group, a MZAPE group, and a radiation plus MZAPE group, and there were 20 rats in each group. The ascites cells of rats bearing with Walker 256 carcinoma were collected, counted and diluted at 2×10^7 /mL, and 0.25 mL was applied to the right axilla of each rat *via* hypodermic inoculation. The experiments were carried out when the tumors grew to a diameter of 0.8-1.0 cm.

A Siemens PRIUS medical electronic linear accelerator (Shandong Tumor Hospital) was used. The radiation dose rate was 100 cGy/min, and the source-skin distance was set at 205 cm. The rats in the simple radiotherapy and radiation plus MZAPE groups underwent radiation with 5 Gy/d of routine radiotherapy for two days (10Gy in total). After 24 h, the MZAPE was gavaged at 16.53 mg/kg once a day for seven days in the MZAPE and radiation plus MZAPE groups. The rats in the simple radiation group and control group were gavaged with 2 mL normal saline for seven days. The rats were sacrificed on the first day after MZAPE administration. Tumor tissues were harvested and weighed, and tumor-inhibiting rate was calculated.

T cell proliferation assay

Spleens were removed on the first day after MZAPE gavage and spleen cell suspensions were prepared. The erythrocytes in the cell suspensions were lysed with Tris-NH₄Cl. A total of 5×10^6 cells/mL in 100 μ L RPMI 1640 containing 1 mmol/L glutamine, 100 U/mL penicillin, 100 μ g/mL streptomycin, 5×10^{-5} mol/L 2-mercaptoethanol and 1% heat-inactivated autologous rat serum were added to each well, followed by the addition of 100 mg/L HEL. The cells were cultured for 72 h. Each well was pulsed with 0.5 μ Ci tritiated thymidine, and the cells were cultured for another 16 h. The cultures were harvested onto fiberglass filters using a multiharvester and counted using standard liquid scintillation techniques.

Measurement of HEL-specific antibodies

Blood was collected on the first day after MZAPE gavage, and sera were heat-inactivated at 56°C for 30 min.

Table 1 Effect of MZAPE on tumor weight, tumor-inhibiting rate, suppression of anti-HEL IgG antibody production, and proliferative responses of PBMC to HEL ($n = 20$, mean \pm SE)

Groups	Tumor weight (g)	Tumor-inhibiting rate (%)	Anti-HEL IgG antibody (A_{405})	Proliferation ($\times 1000$ cpm)
Control	4.27 \pm 0.60		0.17 \pm 0.02	6.0 \pm 0.7
Simple irradiation	1.58 \pm 0.40 ^b	63.08 \pm 6.43	0.09 \pm 0.01 ^b	5.31 \pm 0.8 ^d
Radiation plus MZAPE	1.29 \pm 0.33 ^b	69.86 \pm 7.12	0.34 \pm 0.01 ^b	27.4 \pm 2.8 ^b
MZAPE	2.76 \pm 0.37 ^b	35.30 \pm 7.67	0.44 \pm 0.04 ^b	49.6 \pm 3.1 ^b

^b $P < 0.001$ and ^d $P = 0.006$ vs control group.

IgG, IgG1 and IgG2a antibodies specific for HEL were measured with ELISA (BD Pharmingen, San Diego, CA, USA). In brief, 96-well flat-bottomed microtiter plates were coated with 100 μ L/well HEL (100 mg/L) at 37°C for 1 h and washed three times with PBS. The wells were then blocked by incubation with 100 μ L PBS containing 1% ovalbumin at 37°C for 1 h. After washing, the plates were incubated with 100 μ L of a 1:10000 dilution of each serum sample at 37°C for 30 min. The plates were washed, and 100 μ L/well of a 1:1000 dilution of rabbit anti-rat IgG, IgG1 or IgG2a labeled with alkaline phosphatase was added and incubated at 37°C for 1 h. After washing, 100 μ L of 3 mmol/L p-nitrophenylphosphate was added to each well, and the plates were incubated in the dark at room temperature for 15 min. Absorbance was then measured at 405 nm in a Titertec Multiscan spectrophotometer (EFLAB, Helsinki, Finland). The results were expressed as absorbance units at $A_{405} \pm$ SEM.

Cytokine assays

Single-cell suspensions from spleens were prepared as described above and 5×10^6 cells/mL were cultured in 1 mL aliquots in 24-well tissue culture plates with 100 mg/L HEL. Forty-eight hours later, the supernatants were harvested and stored at -70°C until assayed. The changes in expression of Th1 cytokines (IL-2, INF- γ) and Th2 cytokines (IL-4, IL-10) were detected with ELISA. IL-2, INF- γ , IL-10, IL-4 ELISA sets were purchased from BD Pharmingen (San Diego, CA, USA).

Preparation of lymphocytes

Peripheral blood mononuclear cells (PBMC) were separated from peripheral blood of the rats by Ficoll-Conray method, and the number was counted under microscope. 1×10^6 cells diluted in 1 mL PBS were added to each tube and the phenotypic alternations of the peripheral blood lymphocytes were analyzed by flow cytometry.

FACS analysis

The cells were first stained for surface antigens (30 min at 4°C) with anti-CD3-FITC, anti-CD4-FITC, anti-CD8-FITC, and anti-CD57-FITC (BDIS Biosciences, Stockholm, Sweden). Thereafter, the lymphocytes were permeabilized with FACS-lysing solution and FACS permeabilizing solution (BDIS Biosciences). The staining protocol included isotype controls for both surface and cytoplasmic staining. After staining, the cells were fixed with CellFix (BDIS Biosciences) and acquisition was performed within 2 h. Flow cytometric measurements

Table 2 Effect of MZAPE on anti-HEL IgG2a and IgG1 antibody production ($n = 20$, mean \pm SE)

Groups	Anti-HEL IgG2a antibody (A_{405})	<i>P</i>	Anti-HEL IgG1 antibody (A_{405})	<i>P</i>
Control	0.08 \pm 0.01		0.25 \pm 0.02	
Simple irradiation	0.06 \pm 0.01	< 0.001	0.51 \pm 0.03	< 0.001
Radiation plus MZAPE group	0.15 \pm 0.02	< 0.001	0.396 \pm 0.02	< 0.001
MZAPE group	0.32 \pm 0.04	< 0.001	0.24 \pm 0.01	0.053

were performed using a FACS Calibur (Becton Dickinson, Stockholm, Sweden) and at least 10000 cells/sample were collected. Data analysis was done using Cell Quest software (BDIS Biosciences) according to a standardized pattern-protocol. The background fluorescence was determined with markers applied on the isotype control cytograms and was < 1% in all cases.

Statistical analysis

Statistical analysis was performed using a two-tailed, paired Student's *t* test. Significance was accepted at $P < 0.05$.

RESULTS

Tumor weight and tumor-inhibiting rate

The weight and volume of tumors in the simple radiation, radiation plus MZAPE and MZAPE groups were significantly lower than those in the control group ($P < 0.001$); moreover the tumor-inhibiting rates in the simple radiation, radiation plus MZAPE and MZAPE groups were 63.08% \pm 6.43%, 69.86% \pm 7.12% and 35.30% \pm 7.67%, respectively, (Table 1).

Effect of MZAPE on anti-HEL IgG antibody production, proliferative responses to HEL and anti-HEL IgG2a and IgG1 antibody production in rats

The antigen-specific IgG antibodies in sera, the proliferative responses, anti-HEL IgG2a and IgG1 antibody production of rats immunized with HEL and administered MZAPE were measured on the 8th day. The anti-HEL IgG antibody production markedly decreased in simple irradiation group while increased in radiation plus MZAPE group and MZAPE group when compared to control group ($P < 0.001$). The proliferative responses of PBMC to HEL were significantly enhanced in the radiation plus MZAPE group and MZAPE group compared with the control group ($P < 0.001$). The anti-HEL IgG2a antibody production

Table 3 Effect of MZAPE on peripheral blood CD4 and CD8 cells ($n = 20$, mean \pm SE)

	CD4 ⁺ (%)	<i>P</i>	Lymphocyte subgroups (%)		CD4/CD8	<i>P</i>
			CD8 ⁺ (%)	<i>P</i>		
Control group	38.05 \pm 5.05		19.30 \pm 2.17		1.97 \pm 0.35	
Simple irradiation group	30.36 \pm 2.12	< 0.001 ^b	17.70 \pm 2.34	0.031	1.72 \pm 0.23	0.011
Radiation plus MZAPE group	36.99 \pm 5.24	0.519	17.30 \pm 3.44	0.034	2.13 \pm 0.32	0.140
MZAPE group	32.13 \pm 3.85	< 0.001 ^b	16.70 \pm 1.82	< 0.001 ^b	1.92 \pm 0.26	0.611

^b*P* < 0.001 vs control group.**Table 4** Effect of MZAPE on Th1 and Th2 cytokines ($n = 20$, mean \pm SE) (pg/mL)

	IL-2	<i>P</i>	INF- γ	<i>P</i>	IL-4	<i>P</i>	IL-10	<i>P</i>
Control group	181.99 \pm 19.33		89.06 \pm 21.44		97.69 \pm 7.11		180.14 \pm 19.56	
Simple irradiation group	129.14 \pm 16.96	< 0.001 ^b	74.09 \pm 39.29	0.143	100.57 \pm 12.01	0.362	179.26 \pm 18.13	0.883
Radiation plus MZAPE group	138.16 \pm 23.15	< 0.001 ^b	91.25 \pm 27.00	0.778	74.78 \pm 8.28 ^d	< 0.001 ^{b,d}	180.25 \pm 17.32	0.985
MZAPE group	166.73 \pm 13.34 ^d	0.006 ^b	103.85 \pm 6.55 ^d	0.005 ^b	66.02 \pm 7.14 ^d	< 0.001 ^{b,d}	136.29 \pm 18.21 ^d	< 0.001 ^{b,d}
		< 0.001 ^d		0.002 ^d				

^b*P* < 0.001 vs control group; ^d*P* < 0.001 vs simple irradiation group.

markedly increased in radiation plus MZAPE group and MZAPE group as against the control group ($P < 0.001$). The anti-HEL IgG1 antibody production significantly increased in simple irradiation group and radiation plus MZAPE group ($P < 0.001$). There was no significant change of anti-HEL IgG1 antibody production in MZAPE group (Tables 1 and 2).

Effect of MZAPE on peripheral blood lymphocyte changes of rats

The number of CD4⁺, CD8⁺ lymphocytes in the peripheral blood of the simple irradiation group and the ratio of CD4⁺/CD8⁺ were both decreased. The CD4⁺ lymphocytes in the peripheral blood of the radiation plus MZAPE and MZAPE groups were higher than that in the simple radiation and control groups, and the ratio of CD4⁺/CD8⁺ increased. The CD8⁺ lymphocytes of the radiation plus MZAPE and MZAPE groups showed no significant changes compared to the simple irradiation and control groups (Table 3).

Effect of MZAPE on expression of Th1 (IL-2, INF- γ) cytokines and Th2 (IL-4, IL-10) cytokines in rats

The expression of IL-4 and IL-10 in the spleens of the simple radiation group showed no significant changes, while the expression of IL-2 decreased ($P < 0.001$). In radiation plus MZAPE group, the expression of IL-2 and IL-4 significantly increased ($P < 0.001$). In the MZAPE group, the expression of IL-2, INF- γ , IL-4 and IL-10 significantly increased when compared with the control group and simple radiation group ($P < 0.001$) (Table 4).

DISCUSSION

The male zooid, a kind of bombycidae, is an animal derived medicine in China, and disintegrates from the male tussor chrysalis. According to the Dictionary of Traditional

Chinese Medicine, the major components of the male zooid are proteins, with more than 20 kinds of free amino acids and cytochrome c. Actually, the male zooid also contains various active substances, such as brain hormone, prothymosin, hormone of *Antheraea pernyi*, and diuretics, which can regulate metabolism and restore immune functions^[18]. In addition, such substances may have certain pharmacological activities such as strengthening Yang Qi and astringing essence, promoting host defense mechanisms, and have potentials of anti-aging, anti-tumor and immunity enhancement^[19]. It can be clinically used to treat impotence, seminal emission and stranguria with hematuria. A number of studies have shown the multiple effects of the male zooid in immune responses. The immune system in the Chinese oak silk moth, *Antheraea pernyi*, originated from a single ancestral gene with that of the *Cecropia* moth, whose antibacterial activity has been tested against nine different bacterial species^[20]. Zhang *et al.*^[21] reported that the cecropins from the Chinese oak silkworm *Antheraea pernyi* possess effective anti-tumor activity with no cytotoxicity against normal eukaryotic cells, and impede the neoplastic process in murine large intestines.

It is documented that cytokines such as IL-4 and IL-10 can induce the differentiation of Th1 to Th2 and inhibits the production of Th1 cytokines^[22], affecting immune system and the anti-tumor defense functions. In addition, the immune system of malignant tumor patients could be in immune suppression status and manifest Th2 cell superiority^[23], and radiotherapy could aggravate immunologic injury of the body while it killed tumor cells, resulting in the progressive deterioration of the body's immune function. When the immune function becomes weak, the tumor would easily relapse or transfer to other sites.

In our study, the number of CD4⁺ T cells in the radiated tumor-bearing rats increased after administration of the MZAPE. Such results allow us to hypothesize that

the extracts could strengthen cell immunity by promoting CD4⁺ T cells. In contrast, its effect on CD8⁺ T cells was not obvious and the exact reason for that needs to be investigated in the future. The MZAPE could inhibit the production of Th2 cytokines and reduce the induction of Th2 cytokines in Th1 cells, thereby increasing Th1 cells. The extracts could stimulate the expression of Th1 cytokines, which might induce the transformation of Th0 cells to Th1 cells. We presume that Th1 cells or large number of cytokines secreted by them would activate or enforce immune cell function. Further experiments are needed to elucidate the above-mentioned process. In conclusion, our results demonstrate that MZAPE selectively alters Th1/Th2 cytokine secretion pattern, strengthens immune function of the body and reverses immune suppression induced by radiotherapy. The extracts have a significant effect on enhancing cell immunity by inducing the transformation of Th2 to Th1. This study provides the pharmacological basis for the clinical application of MZAPE.

COMMENTS

Background

Hypoimmunity of tumor patients caused by radiotherapy is one of the major reasons for the failure of treatment and death of patients. Our previous study demonstrated that the concentrated liquor of the male zoid could improve immune function, promote recognition and lethal effects toward tumor cells, and it possessed a unique priority for adjunctive therapy against tumors. In this study the authors evaluated whether supplementation of the male zoid of *Antheraea pernyi* extracts (MZAPE) could enhance the immune function of the radiated tumor-bearing rats.

Research frontiers

The male zoid contains various active substances, which may have certain pharmacological activities such as strengthening Yang Qi and astringing essence, promoting host defense mechanisms, and has potentials of anti-aging, anti-tumor and immunity enhancement. Some studies have shown the male zoid's immunoregulatory function and anti-tumor activity.

Related publications

Our previous study entitled "Immunization of mice with concentrated liquor from male zoid of *Antheraea pernyi*", which was published at World Journal of Gastroenterology, demonstrated that the concentrated liquor of the male zoid could improve immune function, promote recognition and lethal effects toward tumor cells, and it possessed a unique priority for adjunctive therapy against tumors. Other studies also reported that the cecropins from the Chinese oak silkworm *Antheraea pernyi* possess effective anti-tumor activity with no cytotoxicity against normal eukaryotic cells, and impede the neoplastic process in murine large intestines.

Innovations and breakthroughs

The MZAPE administration may improve immune function in radiated tumor-bearing rats and reverse the radiation-induced immune inhibition by promoting the proliferation of T helper cells and inducing the differentiation from Th2 to Th1, and the MZAPE could strengthen the immune function of the body and reverse the immune suppression induced by radiotherapy.

Applications

The MZAPE selectively alters the Th1/Th2 cytokine secretion pattern, strengthens the immune function of the body and reverses the immune suppression induced by radiotherapy. The study provides the pharmacological basis for the clinical application of the male zoid of *Antheraea pernyi* extracts.

Terminology

The male zoid of *Antheraea pernyi* extracts (MZAPE): it has long been used as a pure preparation of traditional Chinese medicine, which possesses many health-

care functions. According to The Great Dictionary of Traditional Chinese Medicine, *Antheraea pernyi* is the matured insect of silkworm.

Peer review

This is an interesting study showing the impact of MZAPE on immune system using a tumor-bearing rat model. The study may benefit from powerful calculation to decide the number of animals needed in each group.

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RAPID COMMUNICATION

Effect of Oxymatrine on the TGFbeta-Smad signaling pathway in rats with CCl₄-induced hepatic fibrosis

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Abstract

AIM: To explore the anti-fibrotic effect of Oxymatrine on CCl₄-induced liver fibrosis in rats and its modulation on the TGFbeta-Smad signaling pathway.

METHODS: One hundred healthy male SD rats were randomly divided into three groups: normal group ($n = 20$), treatment group of Oxymatrine ($n = 40$) and CCl₄-induced fibrosis group ($n = 40$). Experimental hepatic fibrosis was induced by subcutaneous injection of carbon tetrachloride (CCl₄ soluted in liquid paraffin with the concentration of 300 g/L, the dosage of injection was 3 mL/kg, twice per week for 8 wk). The treated rats received Oxymatrine *via* celiac injection at a dosage of 10 mg/kg twice a week at the same time. The deposition of collagen was observed with H&E and Masson staining. The concentration of serum TGF-β1 was assayed with ELISA. The gene expression of Smads and CBP (CREB binding protein) was detected with *in situ* hybridization (ISH) and immunohistochemistry (IH), respectively. All the experimental figures were scanned and analyzed with special figure-analysis software.

RESULTS: A significant reduction of collagen deposition and rearrangement of the parenchyma was noted in the liver tissue of Oxymatrine-treated rats. The semi-quantitative histological scores ($2.43 \pm 0.47 \mu\text{m}^2$ vs $3.76 \pm 0.68 \mu\text{m}^2$, $P < 0.05$) and average area of collagen in those rats were significantly decreased when compared with hepatic cirrhosis model rats ($94.41 \pm 37.26 \mu\text{m}^2$ vs $290.86 \pm 89.37 \mu\text{m}^2$, $P < 0.05$). The gene expression of Smad 3 mRNA was considerably decreased in the treated animals. The *A* value of Smad 3 mRNA was lower in the treated rats than the model rats (0.034 ± 0.090 vs 0.167 ± 0.092 , $P < 0.05$). Contrarily, the *A* value of Smad 7 mRNA was increased considerably in the treated animals (0.175 ± 0.065 vs 0.074 ± 0.012 , $P < 0.05$). There was

an obvious decrease in the expression of CBP mRNA in treated rats as illuminated by a reduction of its *A* value when compared with model rats (0.065 ± 0.049 vs 0.235 ± 0.025 , $P < 0.001$).

CONCLUSION: Oxymatrine is effective in reducing the production and deposition of collagen in the liver tissue of experimental rats. Oxymatrine could promote the expression of Smad 7 and inhibit the expression of Smad 3 and CBP in CCl₄-induced hepatic fibrosis in SD rats, could modulate the fibrogenic signal transduction of TGFβ-Smad pathway.

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Key words: Oxymatrine; Hepatic fibrosis; TGF-Smad signaling

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INTRODUCTION

Several studies have shown that hepatic fibrosis is a reversible disease, therefore an effective treatment would probably prevent or reverse the fibrotic process in the liver^[1]. In the long pathological period of hepatic fibrosis to cirrhosis, transforming growth factor beta 1 (TGFβ1) is one of the strongest pro-fibrotic cytokine^[2,3], and TGFβ-Smad signaling is the cardinal signal transduction pathway^[4] which has been verified by several related studies. The down regulation of TGFβ expression and modulation of TGFβ-Smad signaling may be effective in preventing liver fibrosis^[5]. This study is aimed to explore the anti-fibrotic effect and the probable mechanisms of the extraction of the traditional Chinese medicine, Oxymatrine, in experimental hepatic fibrosis of rats. By examining histopathological changes and deposition of collagen protein in the liver tissue with H&E and Masson

staining, detecting the expression of Smads and CBP with *in situ* hybridization (ISH) and immunohistochemistry (IH), assaying the concentration of serum TGF β 1 with ELISA, we present anti-fibrotic effects of Oxymatrine and discuss the molecular mechanism in an experimental model of carbon tetrachloride (CCl₄)-induced hepatic fibrosis in rats.

MATERIALS AND METHODS

Animals

One hundred healthy male SD rats (weight 140-160 g) were obtained from the Experimental Animal Center of Sichuan University (Chengdu, Sichuan Province, China).

Reagents

Carbon tetrachloride (CCl₄) was obtained from the Chemical and Industrial Reagent Institute in Chengdu. Oxymatrine was from Green Valley Pharmaceutical Co. Ltd., Shanghai, China. Commercial Rat TGF β 1 ELISA Kit and Smad 7 IH Kit were obtained from Boster Biotechnology Co. Ltd., Wuhan, China. The oligonucleotide probe of Smad 3 mRNA (5'→3'): Biotin-GAAGGCCGGCTCACAGTAGGTGACTGGCTG (981-1010 bp, GC% = 63.33), Smad 7 mRNA (5'→3'): Biotin-GAGCTGTCCGAGGCAAAAGCCATTCCTG (2310-2339 bp, GC% = 60.00), and CBP mRNA (5'→3'): Dig-TGACAGTTGTTTATGTTTGGACGC (371-394 bp, GC% = 41.67) were obtained from Shanghai Shenergy Biotechnology Co. Ltd.

Methods

The experimental rats were housed in a room with controlled temperature (15°C-20°C) and lighting (10 h light, 14 h dark). Free access of water and food was allowed during the experimental period. All 100 rats were randomly divided into three groups: Control ($n = 20$), Treatment ($n = 40$) and Model group ($n = 40$). For the model group, 300 g/L CCl₄ soluted in liquid paraffin was injected subcutaneously at a dosage of 3 mL/kg twice per week^[6]. The treated rats received Oxymatrine celiac injections at 10 mg/kg twice a week besides the injection of CCl₄. The injection of CCl₄ and Oxymatrine were without anesthesia. There were no bleeding and other complications after injection. The control group was given normal food and water and received the same dosage and duration of liquid paraffin only as the model group. At the end of the 8-wk experimental period, all the rats were anaesthetized by an intra-muscular injection of sodium pentobarbital (30 mg/kg) before being put to death. Blood was collected from the heart and serum was obtained through centrifugation. The liver was removed rapidly and conserved in 40% neutral formalin for further examination.

Serum concentration of TGF β 1 was detected with enzyme-linked immunoadsorbent assay (ELISA). Liver samples were embedded in paraffin and stained with hematoxylin-eosin (H&E) and Masson collagen staining^[7]. A total of five sections for each liver tissue sample were observed under an optical microscope. The semi-quantitative fibrosis staging scores were acquired according

to the HAI^[8] (histological action index, Table 1): 0: no fibrosis; 1: slight fibrosis, fibrosis located in the central liver lobule; 2: moderate fibrosis, fibrous space formation, but the structure of liver lobule reserved; 3: severe fibrosis, fibrous space enlarged and lobular structure distortion; 4: early cirrhosis or certain cirrhosis, pseudolobule formation.

Each embedded liver sample in paraffin was sliced and fixed onto a poly-L-Lysine covered glass. The gene expression of Smad 3, Smad 7 and CBP mRNA in liver tissues were evaluated with *in situ* hybridization (ISH). The Oligonucleotide probes of Smad 3 and Smad 7 mRNA were marked with biotin at their 5' ends. The Oligonucleotide probe of CBP mRNA was marked with digoxin at its 5' end. The procedure of *in situ* hybridization (ISH) consisted of de-waxing, digesting, pre-hybridizing, hybridizing, coloring, and fixing^[9]. For the color reaction of Smad 3 and Smad 7 mRNA, the NBT/BCIP method was used, and CBP mRNA was detected using DAB. Positive colors of these two methods were purple and brown, respectively. The expression of Smad 7 protein was detected with immunohistochemistry (IH). The procedure of immunohistochemistry (IH) consisted of de-waxing, exposing antigen, repairing antigen, blocking irrespective antigen, combining antigen and antibody, and coloring, respectively. For the color reaction DAB was used, and the positive result were brown particles in the cytoplasm^[10].

Statistical analysis

The quantified markers were counted with mean \pm SD. Slices were scanned under the inverted microscope attached to a computer. The figures were collected and quantified with the software of statistics system. (type TE2000-H, Nikon Ltd, Japan). Random analysis of variance was adopted in the comparison among the different groups, and *t*-test was used in the comparison between different groups.

RESULTS

At the end of experimental period, there were only eighty five rats remained. Five rats in treated group and ten in model group died. Most of the fifteen rats died from injury of biting or being poisoned by Carbon tetrachloride.

Change of TGF β 1 serum concentration

In the control group, the serum concentration of TGF β 1 was only at 1.34 ± 0.25 μ g/L. In the model group, the serum concentration of TGF β 1 was significantly elevated to 3.59 ± 1.23 μ g/L ($P = 0.004$ *vs* control group). It correlated with the semi-quantitative scores of liver fibrosis (the correlation coefficient *r* was +0.59, $P < 0.05$). With the administration of Oxymatrine, the serum concentration of TGF β 1 was decreased significantly to 1.82 ± 0.61 μ g/L ($P = 0.023$ *vs* model group), although it was still higher than the serum concentration of the control group ($P = 0.069$ *vs* control group) (Table 2).

Histopathological changes in the liver

In the control group, the livers showed normal lobular architecture with central veins and radiating hepatic

Table 1 Semi-quantitative scores of hepatic fibrosis staging

Score	Pathological description	Stage
0	No fibrosis	No fibrosis
1	Slight fibrosis	Periportal fibrosis
2	Medium fibrosis	Enlarged periportal fibrosis
3	Severe fibrosis	Bridging fibrosis
4	Cirrhosis	Cirrhosis

Table 2 Serum TGFβ1 concentration and collagen area in liver tissues (mean ± SD)

Group	TGFβ1 (μg/L)	Collagen area (μm ²)
Control	1.34 ± 0.25 ^b	56.12 ± 21.45 ^a
Model	3.59 ± 1.23	290.86 ± 89.37
Treat	1.82 ± 0.61 ^a	94.41 ± 37.26 ^a

^a*P* < 0.05, ^b*P* < 0.01 vs model group.

cords, with 0 staging score. Subcutaneous injection of CCl₄ caused severe hepatic pathological damages such as inflammation, significant hepatic cell necrosis and excessive collagen deposition. The semi-quantitative hepatic fibrosis staging score was raised to 3.76 ± 0.68 in the model group (*P* < 0.01 vs control group). The rats' livers in the Oxymatrine treated group showed less hepatic cells necrosis, less collagen deposition and a significantly decreased staging score of 2.43 ± 0.47 (*P* < 0.05 vs model group) (Table 3, Figure 1 A and B).

Molecular changes of Smad 3, Smad7 and CBP gene expression

There were less positive signals of Smad 3 and CBP mRNA detected with ISH (*in situ* hybridization) in the normal group. The *A* (optical density) value of CBP was nearly 0 (Table 2). In the model group, the positive expression of Smad 3 and CBP mRNA increased significantly. The *A* value of CBP and Smad 3 were increased to 0.235 ± 0.025 and 0.167 ± 0.092 respectively (*P* < 0.01 vs normal group). The treatment with Oxymatrine significantly reduced the *A* value to 0.065 ± 0.049 and 0.034 ± 0.090 (*P* < 0.05 vs model group). At the same time, the positive rate of Smad 7 protein expression was increased from 1.9% to 4.3% (*P* < 0.05 vs model group) (Table 2). Those results demonstrated that Oxymatrine was effective in inhibiting the expression of TGFβ1, Smad 3 and CBP, promoting expression of Smad 7 in the liver (Figures 2 and 3, Table 4).

DISCUSSION

Hepatic fibrosis is thought to be a reversible disease, however, there has not been a satisfied method in the clinical practice to reverse the pathological process yet. Several drugs, including antisense TGFβ receptor, cytokines^[11], antioxidant, chemical drugs, soluble type II receptor of TGFβ1, antibody of TGFβ1 have been used in research work to block experimental hepatic fibrosis, but their effects were not as prosperous as we had expected.

Table 3 Liver histopathological semi-quantitative scores (According to HAI)

Group	<i>n</i>	Scores					Staging scores
		0	+1	+2	+3	+4	
Control	20	20	0	0	0	0	0 ^b
Model	30	0	0	2	3	25	3.76 ± 0.68
Treat	35	0	2	18	13	2	2.43 ± 0.47 ^a

^a*P* < 0.05, ^b*P* < 0.01 vs model group.

Table 4 Expression of Smad 3, Smad 7 and CBP in liver tissues

Group	Smad 7 (IH, %)	mRNA (A)		
		Smad 7	Smad 3	CBP
Control	0 ^b	12:00 AM	0 ^b	0 ^b
Model	0.019 ± 0.002	0.074 ± 0.012	0.167 ± 0.092	0.235 ± 0.025
Treat	0.043 ± 0.009 ^a	0.175 ± 0.065 ^b	0.034 ± 0.090 ^a	0.065 ± 0.049 ^a

^a*P* < 0.05, ^b*P* < 0.01 vs model group.

Besides, some traditional Chinese drugs have been found effective in preventing fibrogenesis and other causes of chronic liver injury^[12,13], and this help to develop a more hopeful future in controlling liver fibrosis and cirrhosis. These drugs have the advantages of being cheap, safe and easy to acquire, but most of them are limited in some animal experiments, rough clinical observation and lack of systemic study at molecular level.

The activation of hepatic stellate cells (HSC) "induced by some critical cytokines" is considered to be of great importance during the long period of liver fibrosis^[14]. This activated HSC then becomes the main source of most cytokines and collagen proteins. Among the cytokines mediating factors, transforming growth factor beta 1 (TGFβ1) has been demonstrated in most research to be an essential pro-fibrogenesis factor^[15-19]. In addition to that, TGFβ-Smad signaling pathway is the main pathway of TGFβ^[20-23], which transfers the stimulating signal from outside into the affected cells. The Smad proteins consists of a large family of transcription factors, which are also found in vertebrates, insects and nematodes. To date, Smads are the only TGF-β receptor substrates with a demonstrated ability to propagate signals. Briefly, two different transmembrane protein serine/threonine kinases, named as TGF-β receptor type I and II respectively, are brought together by the ligand, which acts as a receptor assembly factor^[24]. Before this occurs, receptor I is inactive because a wedge-shaped GS region is inserted into the kinase domain, dislocating the catalytic center. During the TGF-β signal transduction, receptor II is activated firstly. TGF-β and its receptor then form a activated complex. In the ligand-induced complex, activated receptor II phosphorylates the GS region of receptor type I, resulting in the activation of the receptor I kinase. The type I receptors specifically recognize the Smad subgroup known as receptor-activated Smads (R-Smads), which are Smad 2 and Smad 3^[25]. Then R-Smads are activated and forms a complex consisting of R-Smads and Smad 4, which belongs to Co-Smad.

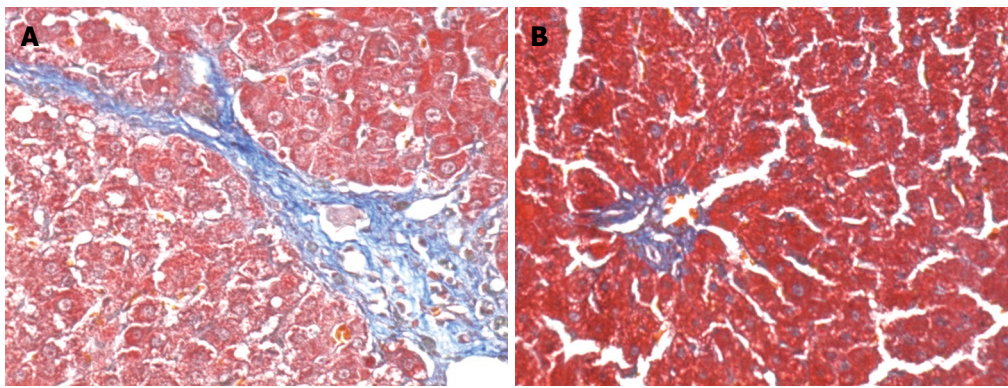


Figure 1 Subcutaneous injection of CCl₄ caused severe hepatic injury such as significant hepatic cell necrosis and excessive collagen deposition. With Masson staining, the collagen fiber was shown blue and hepatic cells were red. (A: Masson, $\times 200$). Oxymatrine treated livers showed less hepatic cell necrosis and less collagen deposition (B: Masson, $\times 200$).

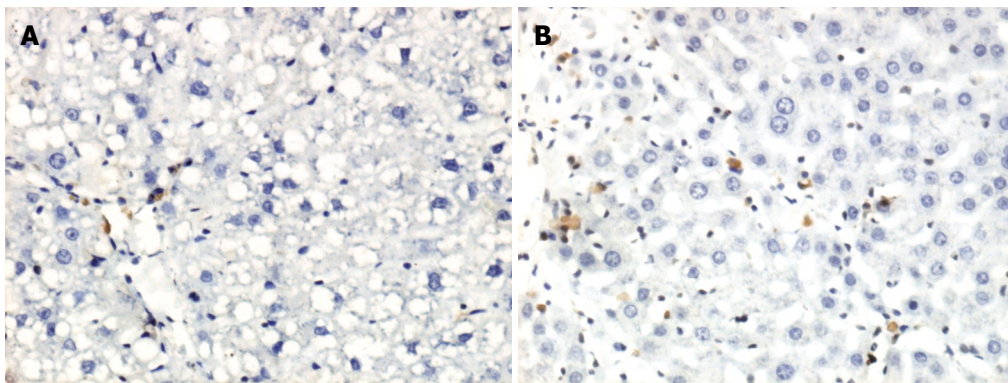


Figure 2 Expression of Smad 7 protein in liver tissue of model rats was increased when detected with immunohistochemistry (A: IH, $\times 200$) when compared to the control group. However, the expression of Smad 7 protein in the livers of the Oxymatrine-treated group was even more significantly increased (B: IH, $\times 200$). The positive rate of Smad 7 protein expression was 1.9% and 4.3% in the model group and the treated group, respectively (data obtained from statistical software, $P < 0.05$).

The Smads-complex then accumulates in the nucleus. This procedure leads to the formation of the functional transcriptional complexes. Both the R-Smads and the Co-Smads in this complex may participate in DNA-binding and recruitment of transcriptional cofactors^[26,27]. CBP (Creb binding protein) is the main downstream molecule and the general transcriptional co-activator. After transferring into the nucleus, the transcriptional complex bind to the certain domain of the target gene and cause the gene expression such as collagen production. The excess collagen production would lead to collagen deposition in liver tissue and hepatic fibrosis or cirrhosis at last. In this pathway, there are two inhibitor Smads (I-Smads), named as Smad 6 and Smad 7, which could combine to the Smads-complex in cytoplasm. Smad 6 and Smad 7 could prevent the Smads-complex to transfer into the nucleus, thus prevent the stimulating signal being transferred from outside into cell nucleus. Since the TGF β -Smad signaling pathway is very important in the formation of hepatic fibrosis, inhibiting the transduction of it may inhibit hepatic fibrosis. As it was shown in some research, inhibiting the TGF β -Smad signaling pathway or modulating the gene expression of certain Smads could interfere with hepatic fibrosis effectively^[28].

Oxymatrine, which is the main component of *Sophora flavescens* Ait, has been used clinically in preventing chronic liver disease^[29,30]. Many studies have shown that it has the effect of protecting hepatocytes, inhibiting the inflammation in liver and reducing the deposition of collagen protein. The present study aimed at exploring the potential mechanisms of Oxymatrine in the prevention of CCl₄-induced hepatic fibrosis in rats.

In this study, chronic administration of CCl₄ caused liver fibrosis and cirrhosis in experimental rats, which is indicated by the histopathological and molecular biological changes in liver tissues. The serum concentration of TGF β 1 in the model group was significantly increased ($3.59 \pm 1.23 \mu\text{g/L}$ *vs* $1.34 \pm 0.25 \mu\text{g/L}$ in the control group, $P < 0.01$), along with the significant collagen deposition. The collagen area was $290.86 \pm 89.37 \mu\text{m}^2$, significantly higher than that of the control group ($56.12 \pm 21.45 \mu\text{m}^2$, $P < 0.05$). Under the optical microscope, the liver fibrosis/cirrhosis was verified by the classical liver structure: damage of liver lobular, hepatic cell necrosis and excessive collagen deposition. Some of the samples have even shown pseudo-lobular formation, which was a pathological symbol of liver cirrhosis. However, with the administration of Oxymatrine, the serum concentration of TGF β 1 was significantly decreased to $1.82 \pm 0.61 \mu\text{g/L}$ ($P < 0.05$ *vs* model group). The HE and Masson stained histopathological slices showed mild necrosis and less collagen deposition. The semi-quantitative fibrosis staging scores were also decreased obviously ($P < 0.01$ *vs* model group). Since the main mechanism of CCl₄-induced liver fibrosis was toxicosis, there was slight inflammation in the livers of both groups. In the normal control group, the expression of Smad 3, Smad 7 and CBP were very low and could hardly be detected. Along with the formation of liver fibrosis, CCl₄ injection also caused an increase in Smad 7, Smad 3 and CBP gene expression, and the expression of Smad 3 and CBP was increased more significantly than Smad 7. We could show that the expression of Smads in liver fibrosis was unbalanced compared to normal liver. The expression of Smad 7 protein and Smad 7 mRNA

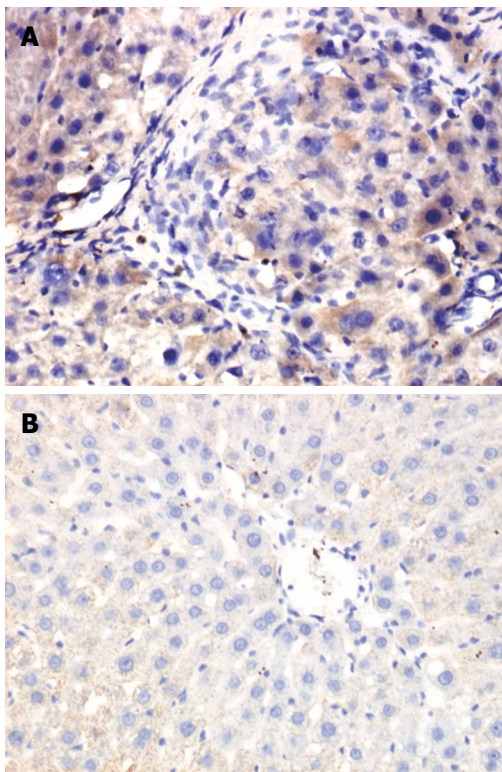


Figure 3 In the control group, the expression of CBP mRNA was at a very low level, the A (optical density) value of it was nearly 0. After CCl₄ injection, the expression of CBP mRNA in the liver of fibrotic rats was significantly enhanced (A: ISH, × 200). However, in the livers of the Oxymatrine-treated rats, the expression of CBP was also increased compared to the control group (B: ISH, × 200), but it was significantly reduced compared to the model group. The A value of CBP mRNA in model group and treated group were 0.235 ± 0.025 and 0.065 ± 0.049 , respectively ($P < 0.05$).

was increased in the Oxymatrine group compared to the model group. The percentage of Smad 7 protein was 0.019 ± 0.002 in the model group, while in Oxymatrine it was 0.043 ± 0.009 ($P < 0.05$ vs model group). The A value of Smad 7 mRNA was 0.074 ± 0.012 in the model group and 0.175 ± 0.065 in Oxymatrine group. The gene expression of Smad 3 mRNA and CBP mRNA were significantly increased in the model group and the O.D. value of them were 0.167 ± 0.092 and 0.235 ± 0.025 , respectively. After Oxymatrine treatment, both Smad 3 and CBP mRNA were inhibited significantly (0.034 ± 0.090 and 0.065 ± 0.049 , both $P < 0.05$) when detected with semi-quantitative evaluation after *in situ* hybridization. However, even with Oxymatrine treatment, Smad 3, Smad 7 and CBP expression remained still significantly increased compared to control group. The detection with *in situ* hybridization and immunohistochemistry could clearly show the change of molecular expression in liver tissue slice. The pathohistological damage of experimental liver could be revealed in the same visual field when the samples were observed under an optical microscope. However, the detection with *in situ* hybridization and immunohistochemistry could not gain an accurate quantity of the molecular expression.

In conclusion, the traditional Chinese medicine Oxymatrine shows significant anti-fibrotic effects in CCl₄-induced liver fibrosis in rats. It can inhibit the expression of Smad 3 and CBP, and promotes the expression of

Smad7. Further studies are needed to explore the exact molecular mechanisms of Oxymatrine in anti-fibrosis.

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RAPID COMMUNICATION

Intraperitoneal administration of gonadotropin-releasing hormone-PE40 induces castration in male rats

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Abstract

AIM: To evaluate the long-term effects of gonadotropin-releasing hormone (GnRH)-based vaccine on levels of GnRH antibody and testosterone, and vaccine-induced immunocastration on sexual behavior of male rats.

METHODS: The rats were treated with GnRH-PE40 intraperitoneally every other day for 12 wk. GnRH antibody and testosterone level in rat blood were determined by ELISA and radioimmunoassay, respectively. Morphological changes in testes and sexual behavior of rats were evaluated.

RESULTS: GnRH-PE40 induced a high production in GnRH antibody, decreased the serum testosterone level, testis atrophy and sexual function in rats.

CONCLUSION: Intraperitoneal administration of GnRH-PE40 produces structural and functional castration of male rat reproductive system by inducing anti-GnRH antibody.

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Key words: Gonadotrophin; *Pseudomonas aeruginosa* exotoxin A; Sexual behavior; Testis atrophy

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INTRODUCTION

Gonadotropin releasing hormone (GnRH) is secreted by the hypothalamus that stimulates the anterior pituitary gland to release gonadotropins in mammals. GnRH has been widely used as a single medical agent^[1] or a conjugated compound with other macromolecules^[2]. Studies indicate that GnRH- based chimerical proteins, consisting of GnRH and toxins, such as *tetanus* toxoid, *diphtheria* toxoid and *pseudomonas aeruginosa* exotoxin A, target specifically at positive tumor cells of GnRH receptor and kill them efficiently^[3-7]. It was reported that some of the chimeric proteins can effectively control reproductive (prostate, breast, ovary and endometrium) and digestive neoplasms^[8-11]. However, since its application in this field, high GnRH antibody titer always develops along with the treatment, which often impedes the use of these compounds^[12,13]. Some authors even reported that the high antibody titer induced by chimeric proteins leads to testis atrophy by depleting immunological hormone^[14].

Male livestock are routinely castrated in most countries to prevent their unpleasant odour (known as boar taint), aggressive behavior and unplanned breeding. As we know, intact male animals have superior feed conversion and leaner carcasses than surgically castrated pigs^[15]. Therefore, the problem is how to concurrently maintain both the intact of animals and the high quality of meat. If the similar strategy of anti-tumor agents mentioned above is applied to contraceptive vaccine, the problem can be possibly solved. Currently, scientists are trying to develop a substitute for the traditional surgical castration. Many preparations based on this theory have been applied to laboratory animals or pets for their immunological castration^[16,17]. It has been demonstrated that immunocastration can improve the meat quality and increase growth performance^[18-20].

GnRH-PE40, one of the recombinant single-chain fusion proteins consisting of GnRH fused to a binding-defective form of *pseudomonas aeruginosa* exotoxin A (PE40), has been developed as a preparation with potential functions of immune castration in male reproductive system. We report here the long term usage of GnRH- based chimeric protein which substantially induces castration in male rat reproductive system.

MATERIALS AND METHODS

Reagents

GnRH-PE40 is a genetic engineering product consisting of PE and GnRH from our laboratory.

Animals

Rats (specific pathogen-free) of Wistar strain, weighing 180–200 g, bought from Animal Center of Military Academy of Medical Sciences (Beijing, PRC), were housed in plexiglass cages (5 per cage) at temperature of 22°C–26°C and humidity of 60% in a 12 h light/dark cycle with free access to food and water. The experimental protocol was approved by the Animal Research Committee of Jinan University.

Treatment procedure

Twenty male rats were randomly divided into treatment group and control group and received intraperitoneal injection of 150 µg/kg of GnRH-PE40 and saline sodium, respectively, every other day for 12 wk. The sexual behaviors of rats were evaluated 12 h after the last injection. The rats were sacrificed under pentobarbital anesthesia 24 h after the last injection. Blood was collected from the heart of comatose rats for hormone or antibody determination. Testes were taken out, weighed, and fixed for histopathological evaluation.

Determination of GnRH antibody by ELISA

A 96-well microtiter plate was coated with 50 µL of 10 µg/mL of GnRH in carbonate bicarbonate buffer (CBB, pH 9.6) overnight at 4°C. After blocked with 3% bovine serum albumin (BSA) in PBS for 1 h at 37°C, the plate was incubated with diluted sera (1:100 to 1:12800) from the rats in different groups in 0.05% Tween 20/PBS with 0.3% (w/v) BSA for 1 h at 37°C. After washing, antibody was detected using horseradish peroxidase (HRP) conjugated goat anti-rat-IgG (BD Pharmingen, San Jose, CA, USA) for 1 h at 37°C. Signals were developed using DAB + substrate (Zhongshan Company, Beijing PRC) and optical density was determined at 490 nm using a BIO-RAD model 550 plate reader. Each measurement of a sample was conducted in duplicate. An absorbance equal to or greater than the mean + 3SE of the control group was considered positive.

Measurement of testosterone

Testosterone level in rat blood was measured by radioimmunoassay using a coat-A-count total testosterone kit (Diagnostic Products Corporation, Los Angeles, USA) according to its manufacturer's instructions. Each measurement of a sample was conducted in duplicate.

Histopathological examination of testis

Testes were fixed in Bouin's solution overnight at 4°C, followed by embedding, sectioning, staining with haematoxylin and eosin, and finally examined histopathologically under light microscope.

Mating behavior test

Ovariectomy was performed for female rats under

Table 1 Anti-GnRH antibody titer, testosterone level and testis weight in rats of the control and treatment groups ($n = 10$)

	Median Ab titer	Testosterone (µg/mL)	Testis weight (g)
Control	1:100	20.3 ± 4.7	4.5 ± 0.9
Treatment	1:1600 ^b	4.7 ± 0.8 ^b	3.1 ± 1.1 ^b

^b $P < 0.01$ vs control group.

ethyl ether anesthesia and 15 µg of estradiol benzoate was subcutaneously injected followed by 500 µg of progesterone 48 h later. Only those exhibiting a good sexual receptivity of male rats, that is, lordosis in response to mounting and with no reject behavior, were used.

The mating behavior of male rats was evaluated during the dark cycle in a sound-proof room, under a dim red light, according to the standard procedure^[21]. After a 10-min adaptation period in a rectangular glass observation cage (60 cm × 50 cm × 40 cm), a stimulus female rat was introduced to a male rat by dropping it gently into the cage. Then following behavioral parameters were recorded or calculated: latency of mount, intromission or ejaculation, and number of mounts, intromissions or ejaculations in a 30-min observation period.

Statistical analysis

Data were expressed as mean ± SE. Where analysis of variance indicated significant differences between groups with ANOVA, for the preplanned comparison of interest, Student's *t* test was applied utilizing the SPSS11.0 version. $P < 0.05$ was considered statistically significant.

RESULTS

GnRH-PE40 induced production of GnRH antibody

ELISA analysis showed that GnRH-PE40 induced a high production of IgG antibody to GnRH in rats. All the animals treated with GnRH-PE40 showed a positive anti-GnRH response. The antibody titers ranged from 1:800 to 1:3200 with a median of 1:1600 (Table 1).

GnRH-PE40 reduced testosterone in rats

Intraperitoneal administration of GnRH-PE40 provoked a significant decrease in blood testosterone of rats. The average testosterone level in rats treated with GnRH-PE40 was 5.4 µg/mL compared with 20.3 µg/mL in control group as shown in Table 1 ($P < 0.01$).

GnRH-PE40 resulted in testis atrophy

Histopathological examination showed that GnRH resulted in remarkable atrophy of testis in rats. The testis weight of rats treated with 150 µg/kg of GnRH-PE40 was decreased by 1.45-fold ($P < 0.01$) (Table 1). The seminiferous epithelium became thinner and the number of spermatogenic cells was decreased compared with that of control group (Figure 1).

GnRH-PE40 enhanced mating performance of male rats

Mating behavior test showed that GnRH-PE40 could improve mating performance of male rats. Compared

Table 2 Influence of GnRH-PE40 intraperitoneal injection on sexual behavior of male rats ($n = 10$, mean \pm SD)

	Latency(s) of			Number		
	Mount	Intromission	Ejaculation	Mount	Intromission	Ejaculation
Control	31 \pm 10	64 \pm 21	605 \pm 107	41 \pm 13	27 \pm 5	2.4 \pm 0.6
Treatment	105 \pm 43 ^b	300 \pm 27 ^b	> 1800	20 \pm 6 ^b	8 \pm 3 ^b	0

^b $P < 0.01$ vs control group.

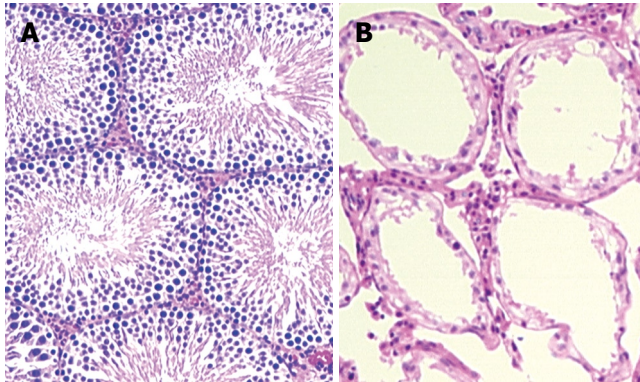


Figure 1 Influence of GnRH-PE40 intraperitoneal injection on spermatogenic cells. **A:** Testes from control rats demonstrating normal seminiferous epithelium (HE, $\times 400$); **B:** Testes from rats treated with 150 μ g/kg GnRH-PE40 demonstrating a significant decrease in spermatogenic cells (HE, $\times 400$).

with the controls, rats that received 150 μ g/kg of GnRH-PE40 exhibited an increased mount and an intromission latency, but a reduced mount and an intromission frequency ($P < 0.01$). No successful ejaculation was observed in rats treated with GnRH-PE40, while the mean ejaculation number in controls was 2.4 in a 30-min observation period (Table 2).

DISCUSSION

GnRH, a short peptide in its natural form, has a poor immunogenicity. When conjugated to a large carrier, it is likely to become an antigen, inducing antibody response. BcePred is a web-based tool for predicting B cell epitope regions in the sequence of an antigen with physicochemical properties. Identified properties of B cell epitope include hydrophilicity, flexibility/mobility, accessibility, polarity, exposed surface and turns. Using this tool, we predicted GnRH motif of GnRH-PE40 with the above properties individually using the default thresholds suggested by software, and found a potential epitope with a higher polarity than the default threshold of 1.8 at GnRH motif^[22]. We hypothesized that this potential epitope was capable of eliciting antibody reaction, which was verified by antibody assay, suggesting that GnRH-PE40 induces a high titer of anti-GnRH antibodies in sera of rats. Hormone determination and histopathological examination showed that intraperitoneal administration of GnRH-PE40 could decrease testosterone level, testis weight and sexual function, which are all indications of testis atrophy^[23-25]. Production of GnRH antibody is possibly the cause for testis atrophy^[26]. It is well-known

that hypothalamus-pituitary-gonadal axis is involved in the control of normal reproductive cycle and maintaining of the structure and function of the reproductive system. Anti-GnRH antibodies reduce the concentration of serum GnRH, which triggers hormone cascades including a lower pituitary LH/FSH and testosterone release, thereby affecting the structure and function of sexual organs^[27]. A high production of anti-GnRH antibodies in serum blocks primarily the function of GnRH, leading to the impairment of sexual organs^[28,29]. Studies showed that anti-GnRH antibodies induce testis atrophy in various laboratory animals and pets immunized with GnRH conjugates^[16,17].

In another study, we showed that 12-wk intravenous administration of GnRH-PE40 to monkeys produced only a low titer of anti-GnRH antibodies, but no marked testis atrophy was found (results not shown here), providing further evidence that antibodies against GnRH are the reason for testis atrophy.

In conclusion, GnRH-PE40 administration produces immunological castration by inducing anti-GnRH antibodies produced in response to the stimulation of B cell epitope of GnRH motif. GnRH-PE40 can be used in animal sciences as a non-surgical castration substitution for surgical castration^[30,31].

COMMENTS

Background

Surgical castration has been widely used as a routine way to prevent unpleasant odour and aggressive behavior of animals. Compared with surgical castration, immunocastration has more advantages such as easy operation in large scale, meat quality improvement and good animal welfare. Therefore, it is necessary to develop new drugs for animal immunocastration.

Research frontiers

Scientists in institutions or pharmaceutical companies are engaged in developing new drugs for animal immunocastration.

Innovations and breakthroughs

A new compound with potential use as an immunocastration agent was found and its physiological properties and actions were studied.

Applications

If the compound reported in this article can be used as an animal immunocastration agent, it will contribute greatly to farmers and production of high quality meat.

Terminology

Castration means surgical removal or artificial destruction of gonads. Immunocastration refers to castration methods based on immunological processes and techniques, such as use of castration vaccines.

Peer review

In the present study, the authors described the treatment of rats with GnRH-PE40,

which resulted in the production of antibodies against GnRH, lower testosterone levels and sexual behavior. The study was well designed and its results strongly support that GnRH can be used as a potential castration agent.

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RAPID COMMUNICATION

Expression of connective tissue growth factor in tumor tissues is an independent predictor of poor prognosis in patients with gastric cancer

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aggressive ability.

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Abstract

AIM: To examine the expression of connective tissue growth factor (CTGF), also known as CCN2, in gastric carcinoma (GC), and the correlation between the expression of CTGF, clinicopathologic features and clinical outcomes of patients with GC.

METHODS: One hundred and twenty-two GC patients were included in the present study. All patients were followed up for at least 5 years. Proteins of CTGF were detected using the PowerVision two-step immunostaining method.

RESULTS: Of the specimens from 122 GC patients analyzed for CTGF expression, 58 (58/122, 47.5%) had a high CTGF expression in cytoplasm of gastric carcinoma cells and 64 (64/122, 52.5%) had a low CTGF expression. Patients with a high CTGF expression showed a higher incidence of lymph node metastasis than those with a low CTGF expression ($P = 0.032$). Patients with a high CTGF expression had significantly lower 5-year survival rate than those with a low CTGF expression (27.6% vs 46.9%, $P = 0.0178$), especially those staging I + II + III (35.7% vs 65.2%, $P = 0.0027$).

CONCLUSION: GC patients with an elevated CTGF expression have more lymph node metastases and a shorter survival time. CTGF seems to be an independent prognostic factor for the successful differentiation of high-risk GC patients staging I + II + III. Over-expression of CTGF in human GC cells results in an increased

INTRODUCTION

Gastric cancer (GC), one of the most common malignant diseases, is the second leading cause for cancer-related death both in China and in the world (700 000 deaths annually)^[1,2].

TNM staging system is used worldwide to predict the prognosis and direct therapeutic decisions of patients with GC^[3]. The 5-year survival rate of GC patients at stages I and IV is close to 90% and less than 30%, respectively^[4]. GC exhibits markedly heterogeneous in histologic feature and biologic behavior, especially at advanced stages. It was reported that the biological behavior and prognosis of GC can be significantly different among GC patients at the same stage^[5]. Some studies showed that some biomarkers could provide additional information for predicting the biological behavior and prognosis of GC. More specific and effective markers and therapies should be identified and developed for improving the survival of GC patients.

Connective tissue growth factor (CTGF), also known as CCN2, is a member of the CCN family, including cysteine-rich protein 61 (Cyr61), also known as CCN1, and nephroblastoma-overexpressed gene (Nov), also known as CCN3, as well as Wisp-1/elm1 (CCN4), Wisp-2/rCop1 (CCN5) and Wisp-3 (CCN6)^[6,7]. The primary translational products of CCN family members are 343-381 residues, which generate proteins of Mr 35 000-40 000 with homologies ranging from 60% to 90%.

All members of the CCN gene family possess a secretory signal peptide at the NH₂ terminus, indicating that they are secreted proteins. CTGF can bind to integrins on cell surface^[6], and is a potent stimulator of endothelial cell adhesion, proliferation, migration and angiogenesis *in vivo*^[9-11]. CTGF is believed to be a multifunctional signaling modulator involved in a wide variety of biologic or pathologic processes, such as angiogenesis, osteogenesis, fibrosis in kidneys and skin, and tumor development^[6-8,12-15]. It was reported that CTGF plays an important role in the progression of several types of cancer^[16]. Elevated CTGF levels have been detected in a number of cancers including pancreatic cancer^[16,17], breast cancer^[18,19], prostate cancer^[20], esophageal adenocarcinoma^[21], glioma^[22] and melanoma^[23]. However, little information on the association between expression of CTGF and GC prognosis is available.

In this study, we examined the expression of CTGF in gastric carcinoma in order to analyze its correlation with histologic type, clinicopathologic feature, and clinical outcome of gastric carcinoma patients.

MATERIALS AND METHODS

Patients and tissue samples

A consecutive series of 122 patients with gastric carcinoma were studied. All patients were treated at the Department of Surgery, Affiliated Hospital of Binzhou Medical College, between July 1994 and December 2000. All patients gave their written informed consent to participate in this study. There were 88 males and 34 females with a mean age of 56.6 years (range 25-80 years). All patients underwent radical gastrectomy and none of the patients received chemotherapy or radiation therapy prior to operation. Age and sex of the patients, maximum tumor size, histologic grade, status of lymph node metastasis and distant metastasis were obtained from histopathology reports. Stage of GC was defined according to the 1997 tumor-node-metastasis (TNM) classification of malignant tumors by the International Union against Carcinoma^[24]. All patients were followed-up until May 2007.

Immunohistochemistry

The tissue, fixed in 10% neutral formalin and embedded in paraffin, was cut into 4- μ m thick sections. CTGF expression was examined by immunostaining using the PowerVision two-step immunostaining method. Briefly, the sections were treated with a 3% hydrogen peroxide solution for 10 min to block the endogenous peroxidase activity after deparaffinized in xylene and rehydrated in a graded ethanol series. Antigen retrieval was performed in 1 mmol/L EDTA (pH 8.0) in an autoclave for 3 min. The monoclonal antibodies used were clone 88430 (1:100, R&D Systems Inc, Minneapolis, MN, USA) which recognizes CTGF. The sections were incubated overnight at 4°C with primary antibody. The primary antibody was detected using the PowerVision two-step histostaining reagent-peroxidase-labeled goat anti-mouse immunoglobulin (PV-6002, DAKO, Glostrup, Denmark) for 1 h at room temperature. After peroxidase activity was developed with 3, 3'-diaminobenzidine tetrachloride (DAB), slides were counterstained with haematoxylin and

observed under a light microscope. Positive and negative immunohistochemistry controls were routinely used.

Three experienced pathologists, unaware of the information on the clinicopathologic data and clinical outcomes of the patients, independently examined the CTGF staining. A scoring system was devised to assign a staining intensity score for CTGF expression from 0 (no expression) to 3 (highest intensity staining). Immunostaining was classified into two groups according to both intensity and extent. Low expression was defined as no staining present (staining intensity score: 0) or positive staining detected in $\leq 10\%$ of the cells (staining intensity score: 1) and high expression was defined as positive immunostaining present in 10%-50% of the cells (staining intensity score: 2) or $> 50\%$ of the cells (staining intensity score: 3)^[25].

Statistical analysis

All data were analyzed using SPSS 10.0 software. The association of CTGF expression with various clinicopathologic features was analyzed using the Pearson χ^2 test. Cumulative survival was estimated with the Kaplan-Meier method and the difference in survival curves was analyzed by the log-rank test. The influence of each variable on survival was analyzed with the multivariate analysis of Cox proportional hazard model (backward, stepwise). All statistical tests were two-sided. $P < 0.05$ was considered statistically significant.

RESULTS

Patients

The clinicopathologic features of the patients are summarized in Table 1. The follow-up time ranged from 2 mo to 121 mo (median, 27 mo). The 5-year survival rate of patients at stages I, II, III and IV was 88.9%, 66.7%, 28.3% and 2.9%, respectively. The overall 5-year survival rate was 37.7%.

CTGF expression in gastric carcinoma

The CTGF protein was predominantly localized in cytoplasm or membrane of normal or tumor cells. No CTGF expression was detected in normal gastric epithelial cells, but deep glands and fibroblasts were positively stained. Glands in some cases were positively stained in intestinal metaplasia and dysplasia gastric mucosa.

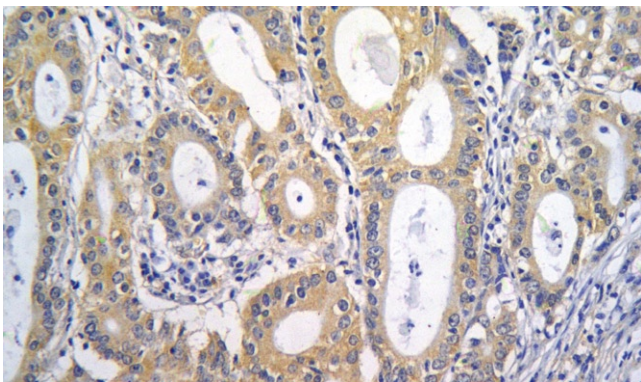
Of the 122 specimens from GC patients analyzed for CTGF expression, 58 (58/122, 47.5%) had a high CTGF expression in cytoplasm of gastric carcinoma cells, 43 (43/122, 35.2%) had a score of 2, and 15 (15/122, 12.3%) a score of 3, while 64 (64/122, 52.5%) had a low CTGF expression, 37 (37/122, 30.3%) had a score of 0 and 27 (27/122, 22.1%) a score of 1 (Figure 1).

CTGF expression in relation to clinicopathologic features of gastric carcinoma

CTGF was highly expressed more frequently in well-differentiated GC than in moderately- or poorly-differentiated GC ($P = 0.014$) and in intestinal-type carcinoma than in diffuse-type or mixed-type carcinoma ($P = 0.045$). Patients with a high CTGF expression had

Table 1 Association between CTGF expression and clinico-pathologic factors

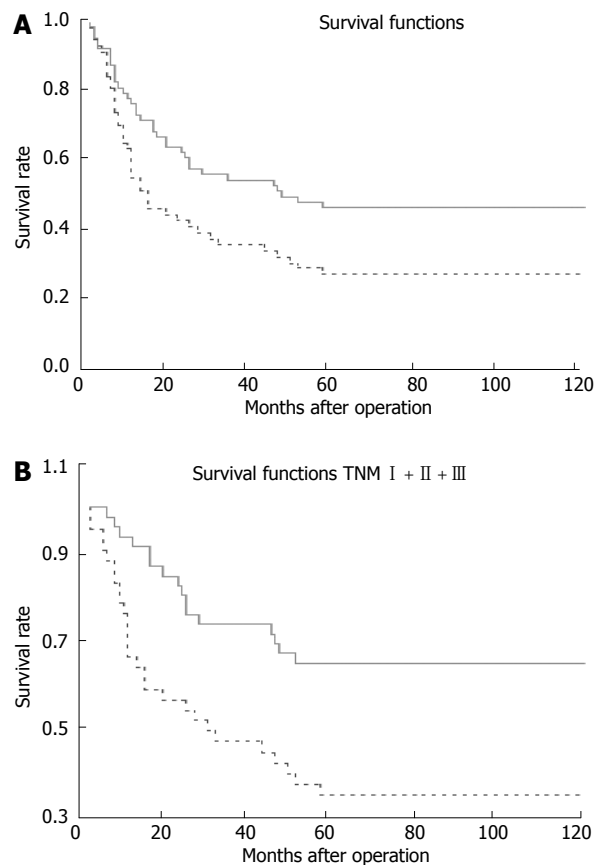
Factors	Cases	CTGF expression		P value ¹
		Low expression	High expression	
Age (yr)				0.628
< 60	68	37	31	
≥ 60	54	27	27	
Sex				0.251
Male	88	49	39	
Female	34	15	19	
Tumor size (cm)				0.555
< 5	56	31	25	
≥ 5	66	33	33	
Differentiation				0.014
Well	19	6	13	
Moderate	32	13	19	
Poor	71	45	26	
Lauren type				0.045
Intestinal type	40	15	25	
Diffuse type	64	40	24	
Mixed type	18	9	9	
TNM stage				0.391
I	18	11	7	
II	24	15	9	
III	46	20	26	
IV	34	18	16	
Lymph nodes metastasis				0.032
Absent	32	22	10	
Present	90	42	48	
Metastasis				0.821
Absent	104	55	49	
Present	18	9	9	

¹Pearson χ^2 test.**Figure 1** Immunohistochemical staining for connective tissue growth factor (CTGF) in gastric carcinoma (× 400).

a higher incidence of lymph node metastasis than those with a low CTGF expression ($P = 0.032$). No significant relationship was found between the level of CTGF expression and the age and sex, tumor size, TNM stage and distant metastasis of GC patients (Table 1).

Univariate analysis of prognostic impact of CTGF expression on gastric carcinoma

Patients with a high CTGF expression had a significantly lower cumulative 5-year survival rate (27.6%) than those with a low CTGF expression (46.9%, two-sided log-rank

**Figure 2** Kaplan-Meier survival curves for patients with a low (—) or a high (---) expression of CTGF (A) and for those at stage I + II + III with a low (—) or a high (---) expression of CTGF (B). The survival of patients with a low CTGF expression was significantly longer than those with a high CTGF expression, $P = 0.0178$ (A) and $P = 0.0027$ (B), respectively.

test, $P = 0.0178$; Figure 2A). The prognostic significance of CTGF expression in patients at TNM stage I + II + III was analyzed. Patients at stage I + II + III had a high CTGF expression and a significantly lower 5-year survival rate (35.7%) than those with a low CTGF expression (65.2%, two-sided log-rank test, $P = 0.0027$; Figure 2B).

Multivariate analysis of prognostic impact of CTGF expression on gastric carcinoma

Multivariate analysis revealed that CTGF expression, TNM stage, differentiation were independent prognostic indicators for the overall survival of the patients after adjustment for sex, age, tumor size, grade of differentiation, Lauren types, TNM stages, lymph node metastasis and distant metastasis ($P < 0.05$, Table 2).

DISCUSSION

In the present study, we detected CTGF expression in GC patients. High CTGF expression was closely related with lymph node metastasis, grade of differentiation, and Lauren type. Univariate and multivariate analyses revealed that high CTGF expression was a powerful independent predictor for the poor survival of GC patients, especially for those at stage I + II + III. The overall 5-year survival rate of GC patients with a higher CTGF expression and a

Table 2 Multivariate analysis of the prognostic impact of CTGF expression by Cox proportional hazard model with backward stepwise procedure

Variables	B	SE	RR (95% CI)	P
TNM stage				< 0.001
II vs I	1.162	0.792	3.197 (0.677-15.099)	0.142
III vs I	2.202	0.734	9.039 (2.143-38.136)	0.003
IV vs I	3.561	0.746	35.208 (8.165-151.830)	< 0.001
Differentiation				0.067
Moderate vs Well	0.771	0.381	2.162 (1.024-4.567)	0.043
Poor vs Well	0.929	0.414	2.533 (1.126-5.699)	0.025
CTGF expression				
High vs Low	0.565	0.265	1.760 (1.047-2.958)	0.033

B: Coefficient; RR: Relative risk; CI: Confidence interval.

lower CTGF expression was 27.6% and 46.9%, respectively ($P = 0.0178$). The 5-year survival rate of GC patients with a higher CTGF expression and a lower CTGF expression at stage I + II + III was 35.7% and 65.2%, respectively ($P = 0.0027$), indicating that over-expression of CTGF could promote the aggressive behavior of GC.

CTGF is a novel, potent angiogenic factor^[9,10], which was first identified as a mitogen, detected in conditioned medium from human umbilical vein endothelial cells^[26]. Integrin is an important receptor for CCN proteins, and receptor activation may produce a variety of effects. CTGF protein can bind directly to integrins $\alpha v \beta 3$ and $\alpha II b \beta 3$ ^[10,11]. Shimo *et al*^[9] and Babic *et al*^[10] reported that CTGF mediates endothelial cell adhesion and migration through binding to integrin $\alpha v \beta 3$, prolong endothelial cell survival, and induce angiogenesis *in vivo*. Yang *et al*^[20] reported that CTGF is a downstream mediator of TGF- $\beta 1$ action in cancer-associated reactive stroma, and one of the key promoters of angiogenesis in tumor-reactive stromal microenvironment, and plays an important role in prostate carcinogenesis. Breast cancer stage is positively associated with tumor size, lymph node metastasis status and over-expression of CTGF^[19]. In our study, high CTGF expression was related with lymph node metastasis, depending on the ability of CTGF to induce angiogenesis.

CTGF is believed to be a multifunctional signaling modulator involved in a wide variety of biologic or pathologic processes. CTGF proteins exhibit diverse cellular functions, such as regulation of cell division, proliferation, mitogenesis, differentiation, survival, adhesion and migration, apoptosis, motility, and ion transport. CTGF plays a role in the development and progression of cancer. Recently, Dornhöfer *et al*^[16] showed that CTGF promotes anchorage-independent pancreatic cancer cell growth. Furthermore, anti-CTGF treatment inhibits anchorage-independent growth *in vitro*, primary tumor growth *in vivo* and macroscopic lymph node metastases^[16]. In contrast to the above results, CTGF is a new autocrine survival and differentiation factor for human rhabdomyosarcoma cells^[27]. It was reported that over-expression of CTGF suppresses the growth of oral squamous carcinoma cells transplanted into mice^[28]. Furthermore, apoptosis of MCF-7 cells induced by TGF- β appears to be mediated by CTGF, suggesting that CTGF may play an important role in

human breast cancer cell growth^[29]. Elevated level of CTGF is significantly correlated with a good prognosis of colorectal cancer^[30] and lung adenocarcinoma^[25], suggesting that the role of CTGF in different types of cancer may vary considerably, depending on the tissue involved. The question of how cell or tissue context determines the action of CTGF protein is interesting and deserves further investigation.

The present study showed that high CTGF expression was a powerful independent predictor for the poor overall survival of GC patients, especially for those at stage I + II + III. Multi-mechanisms are involved in aggressive behaviors of tumors at stage IV. The 5-year survival rate was only about 10% of GC patients at stage IV. Additional biomarkers might be helpful in predicting the prognosis of GC patients and more specific and effective therapies should be developed to improve the survival of GC patients at stage I + II + III. However, the value of additional biomarkers for predicting the prognosis of GC patients at stage IV is poor.

In conclusion, GC patients with an elevated CTGF expression have more lymph node metastases and a shorter survival time. CTGF seems to be an independent prognostic factor that allows successful differentiation of high-risk GC patients at stage I + II + III. Over-expression of CTGF in human GC cells results in an increased aggressive ability of cancer.

ACKNOWLEDGMENTS

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COMMENTS

Background

Connective tissue growth factor (CTGF), also known as CCN2, is a member of the CCN family, which is believed to be a multifunctional signaling modulator involved in a wide variety of biologic or pathologic processes. CTGF plays an important role in the progression of several types of cancer. However, little information on the association between CTGF expression and GC prognosis is available.

Research frontiers

In this study, we examined the expression of CTGF in gastric carcinoma in order to analyze its correlation with histologic type, clinicopathologic feature, and clinical outcomes of gastric cancer (GC) patients.

Innovations and breakthroughs

GC, one of the most common malignant diseases, is the second leading cause for cancer-related death both in China and in the world. It has been shown that its biologic behavior and prognosis can be significantly different in GC patients at the same stage. CTGF seems to be an independent prognostic factor that allows differentiation of high-risk patients at stage I + II + III. Over-expression of CTGF in human GC cells results in an increased aggressive ability of GC.

Applications

CTGF may represent a potential novel target for treatment of GC. Inhibition of CTGF may control primary tumor growth and lymph node metastasis.

Peer review

In this study, the authors showed that CTGF was a prognostic factor for GC patients. This paper is well-written.

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Endoscopic ultrasonography-guided trucut biopsy for the preoperative diagnosis of peripancreatic castleman's disease: A case report

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INTRODUCTION

Castleman's disease (CD) of the pancreas/peripancreas is a very uncommon disease. The differential diagnosis of peripancreatic masses includes pancreatic cancer, tuberculous lymphadenopathy, lymphoma, neurogenic tumors such as paraganglioma, and CD. The initial challenge in CD is to establish its correct diagnosis. Radiology-guided fine needle aspiration (FNA) of deep-seated masses is a standard means of obtaining a tissue diagnosis, but fails to make a definitive distinction from lymphoma^[1]. Recently, the efficacy of endoscopic ultrasonography (EUS)-guided TCB in diagnosing intra-abdominal/retroperitoneal benign lymph node enlargement or lymphoid malignancies has been reported. Herein, we present the EUS morphologic findings, microscopic examinations, and the advantage of polymerase chain reaction (PCR) for IgH gene rearrangement in a case of peripancreatic CD which was initially sampled by EUS-TCB.

CASE REPORT

A previously healthy, 50-year-old woman was referred to our institution for further evaluation of a pancreatic mass which was incidentally detected on an abdominal computed tomography (CT) scan performed at another hospital.

Dynamic CT scans of her pancreas disclosed an arterial, enhancing mass measuring 3.9 cm in the upper portion of the pancreas body (Figure 1). EUS (GF-UM2000; Olympus, Japan) demonstrated a homogenous, elongated, and well-delineated hypoechoic mass between the left lobe of liver and the body of the pancreas. The vascularity of the mass was high on Doppler US (Figure 2). A core biopsy specimen of the lesion was obtained using a 19-gauge, trucut needle (EUSN-19-QC, Cook) under the guidance of a linear-array echoendoscope (GF-UCT2000; Olympus, Japan). After the procedure, the patient had no immediate complications, such as bleeding, perforation or peritonitis. The size of the core biopsy specimen was about 1.1 cm in length, but it was fragmented into five

Abstract

Castleman's disease (CD) of the pancreas/peripancreas is extremely rare. The recently introduced, endoscopic ultrasonography (EUS)-guided trucut biopsy (TCB) is a useful diagnostic modality for obtaining tissue samples from peripancreatic lesions. However, its role in diagnosing CD remains unknown. We report a case of localized, peripancreatic, hyaline-vascular CD biopsied using EUS. The pathology results were initially interpreted as an extranodal, marginal-zone B-cell lymphoma. However, polymerase chain reaction (PCR) study for the IgH gene rearrangement revealed a polyclonal pattern. We also reviewed the relevant literature. To our knowledge, this is the first illustrated report on EUS-TCB findings of CD with its pathology results of EUS-TCB mimicked a B-cell lymphoma.

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Key words: Castleman's disease; Endoscopic ultrasonography; Biopsy; Lymphoma; Diagnosis

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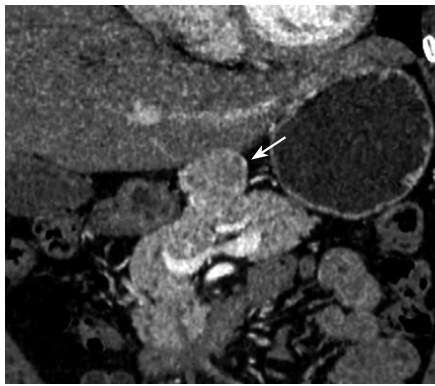


Figure 1 Pancreas dynamic CT scan showing a well-demarcated, arterial, enhancing mass measuring 3.9 cm (arrow) just above the pancreatic body.

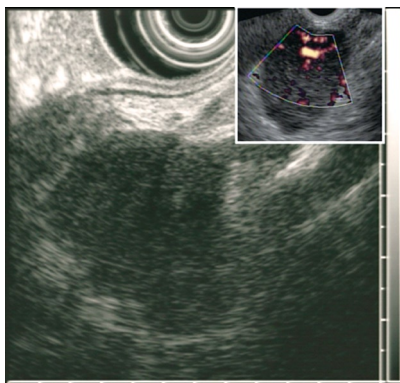


Figure 2 Endoscopic ultrasonography showing a well-delineated, homogenous, elongated mass with a hypervascular appearance on Doppler (small box).

pieces while putting into a tissue container. Microscopic examination showed expanded follicles replaced by dense infiltration of small, mature lymphocytes devoid of follicular center cells which caused an initial suspicion of low-grade, extranodal, marginal-zone B-cell lymphoma. However, according to PCR study for IgH gene rearrangement, the mass was seen to have a polyclonal pattern (Figure 3A and B).

The patient underwent laparoscopic mass excision with curative intent as well as pathologic confirmation. The resected lesion was found to be a well-demarcated, lobulated, rubbery, firm mass (Figure 4A). Microscopically, the lesion was a markedly enlarged lymph node composed of numerous, prominent, lymphoid follicles with atrophic germinal centers devoid of follicle center cells (Figure 4B).

The final diagnosis was CD (angiofollicular lymph node hyperplasia) of a hyaline-vascular (HV) variant. The patient had an uneventful postoperative course and remained disease-free at the time when this report was prepared.

DISCUSSION

CD has been identified in a series of patients with solitary, hyperplastic, mediastinal lymph nodes with small germinal centers resembling Hassall's corpuscles of the thymus^[2]. CD can be divided into hyaline vascular type, plasma cell type, and mixed type or into unicentric or localized type and multicentric or generalized type^[3,4]. The HV type accounts for 90% of all localized disease^[5] and is often asymptomatic or, as in our patient, has symptoms caused by the mass effect of the lesion.

The clinical challenge in CD is to establish its correct

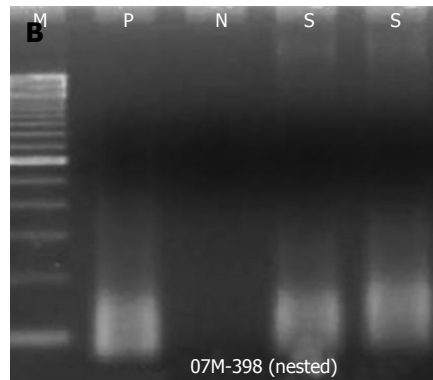
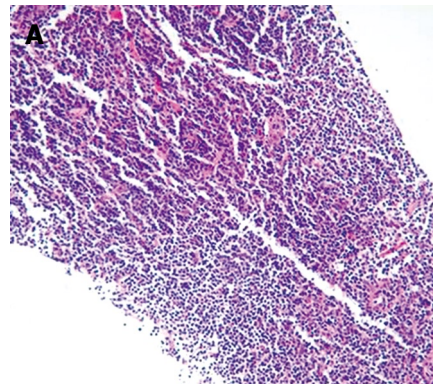


Figure 3 Microscopic examination of the EUS-TCB specimen showing expanded follicles replaced by a dense infiltration of small, mature lymphocytes devoid of follicular center cells (A) (× 200) and PCR study for IgH gene rearrangement showing a polyclonal pattern (B).

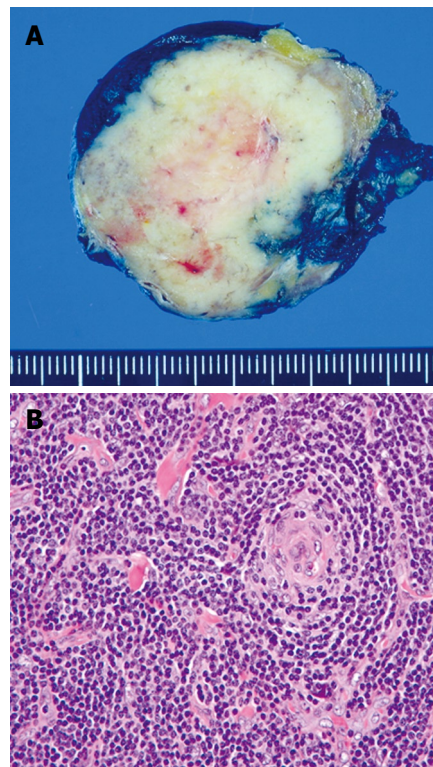


Figure 4 Surgical specimen showing creamy white cut surface of the resected tumor with trabeculation and numerous, small blood vessels (A) and lymph node demonstrating burnt-out, atrophic follicles with depletion of follicle center cells and residual, follicular, dendritic cells and surrounding, concentric layers of small lymphocytes (B). Radial penetrating blood vessels are also seen (× 400).

diagnosis. Many imaging modalities, including CT, magnetic resonance (MR), angiography and FDG-PET, have been used to diagnose CD^[6-9]. Nevertheless, it is the mainstay to obtain and analyze tissue samples for its final diagnosis.

EUS-, US- or CT- guided FNA of deep-seated masses are the standard means of obtaining tissue diagnosis, but cytomorphologic, flow cytometric, and

immunohistochemical studies have failed to make a definitive distinction from certain kinds of lymphoma^[1]. The reason is that FNA specimen is largely restricted in morphologic and immunohistochemical study for the shortage of sample size. On the other hand, EUS-TCB has a higher diagnostic yield and accuracy because it may obtain more adequate core specimens than FNA. It was reported that EUS-TCB has been used in diagnosing gastrointestinal stromal tumor (GIST), leiomyoma, and lymphoma^[10,11].

Although the initial microscopic morphology of our case was similar to that of B-cell lymphoma, lymphoma was mostly excluded and CD was strongly suspicious prior to surgery with the help of PCR for IgH gene rearrangement.

In conclusion, peripancreatic CD is rare but should be considered in the differential diagnosis of peripancreatic lesions. The discrimination of CD from lymphoma could be difficult, but we believe that EUS-TCB is more useful in preoperative diagnosis than FNA.

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CASE REPORT

Primary rectal signet ring cell carcinoma with peritoneal dissemination and gastric secondaries

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Abstract

Disseminated signet ring cell carcinomas frequently arise from the stomach. However, primaries in the colon and rectum have also been reported. We present a 68 year old lady who presented with a change in her bowel habit. Colonoscopy showed a stenosing rectal tumour at 7 cm to 8 cm from the anal verge. Multiple scattered ulcers were also noted along the entire length of the colon. Biopsy of the lesions revealed signet ring cell adenocarcinoma. Gastroscopy showed multiple nodules with ulceration over several areas of the stomach which were similar in appearance to the colonic lesions. However, no primary tumour of the stomach was seen. Biopsy of the gastric lesions also showed signet ring cell adenocarcinoma. Computed tomography scan of the abdomen and pelvis revealed circumferential tumour at the rectosigmoid junction with possible invasion into the left ischiorectal fossa. The overall picture was that of a primary rectal signet ring cell carcinoma with peritoneal dissemination. The patient was referred for palliative chemotherapy in view of the disseminated disease. In the present report, we discuss this interesting pathological entity and review the role of various histological techniques in helping to identify the primary tumor.

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Key words: Signet ring cell carcinoma; Colorectal tumour; Peritoneal dissemination; Gastric secondaries

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Sim HL, Tan KY, Poon PL, Cheng A. Primary rectal signet ring cell carcinoma with peritoneal dissemination and gastric secondaries.

INTRODUCTION

Primary colorectal signet ring cell carcinoma is a rare but distinctive malignancy of the large bowel. More than 96% of the signet ring cell carcinomas arise in the stomach, with the remainder arising from other sites, including colon, rectum, gallbladder, pancreas, urinary bladder and breast^[1]. Therefore, metastasis to the colon and rectum must be excluded before a definite diagnosis of primary colorectal signet-ring cell carcinoma can be made.

CASE REPORT

A 68-year-old female patient with history of diabetes mellitus and hypertension presented with one month history of change in bowel habit. The patient complained of increased frequency of stools, with the passage of only small amounts of stools each time. There was no history of rectal bleeding, or loss of appetite or weight. Rectal examination revealed a hard mass about 6 cm from the anal verge.

Colonoscopy showed a hard stenosing rectal tumour at 7 cm to 10 cm from the anal verge (Figure 1A and B). Multiple scattered ulcers were found throughout the colon from the caecum to the sigmoid colon. Biopsy of the rectal tumour and the ulcers were performed. Transrectal ultrasound revealed a UT3N1 tumour of the mid-rectum.

Carcino-embryonic antigen level was 10.7. Computed tomography of the abdomen and pelvis showed a circumferential tumour in the recto-sigmoid junction with no evidence of hepatic metastases or intrabdominal lymphadenopathy or a second primary.

Histopathology of the colonic ulcers and the rectal tumour showed large sheets and aggregates of signet ring cells with foamy cytoplasm and pleomorphic, hyperchromatic eccentric nuclei (Figure 2A). The tumour cells infiltrated between the mucosal glands. Areas of ulceration and focal mucin pools were noted. These appearances were consistent with signet ring cell adenocarcinoma.

In view of the histological finding of signet ring cell adenocarcinoma, gastroscopy was performed to exclude a gastric primary tumor. Gastroscopy revealed multiple nodules with ulceration scattered over body, incisura and

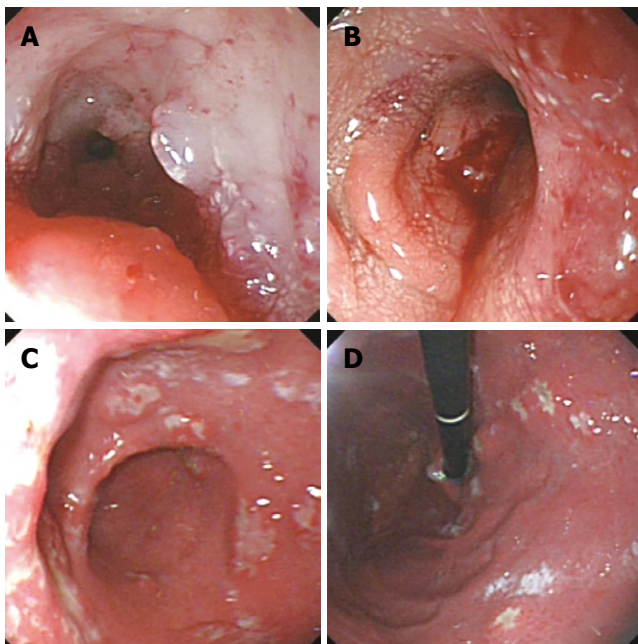


Figure 1 Colonoscopic pictures (A) and (B) show stenosing primary rectal tumour with multiple ulcerations; Gastroscopy (C) and (D) showed multiple ulcers in the stomach.

antrum of the stomach (Figure 1C and D). No primary gastric tumour was found. Biopsy of the lesions showed signet ring cell adenocarcinoma similar to that found in the rectum and colon.

The clinical picture was that of a primary rectal signet ring cell adenocarcinoma with peritoneal dissemination and gastric metastasis. Immunohistochemical studies were performed to differentiate between a gastric and colonic primary tumor. Both specimens were positive for cytokeratin 7 (CK 7) (Figure 2B) and Cytokeratin 20 (CK 20).

In view of the disseminated peritoneal disease and minimal symptoms, the patient was not offered surgery. She received palliative chemoradiation and was clinically well at 6 mo follow-up.

DISCUSSION

Primary signet ring cell carcinoma of the colon and rectum is a rare variant of colorectal adenocarcinomas. The incidence of signet ring cell carcinoma is reported to range between 0.1% and 2.4%^[2,3]. The diagnosis of primary colorectal signet ring cell carcinoma is often based on the histological appearance of the specimen with exclusion of a gastric primary.

Signet ring cell tumours have an aggressive clinical course and a poor prognosis^[4-6]. There is high incidence of peritoneal metastases and relatively low incidence of hepatic metastases, a characteristic feature distinguishing colorectal signet-ring cell carcinoma from non-signet colorectal carcinoma.

According to Secco *et al*, the 5-year survival rate of primary colorectal signet ring cell carcinoma was 0% (median 15 mo) and disease recurrence was 100%. However, a local study from Singapore reported 5-year survival rate of 12%^[7]. Generally, the prognosis of signet-ring cell carcinoma

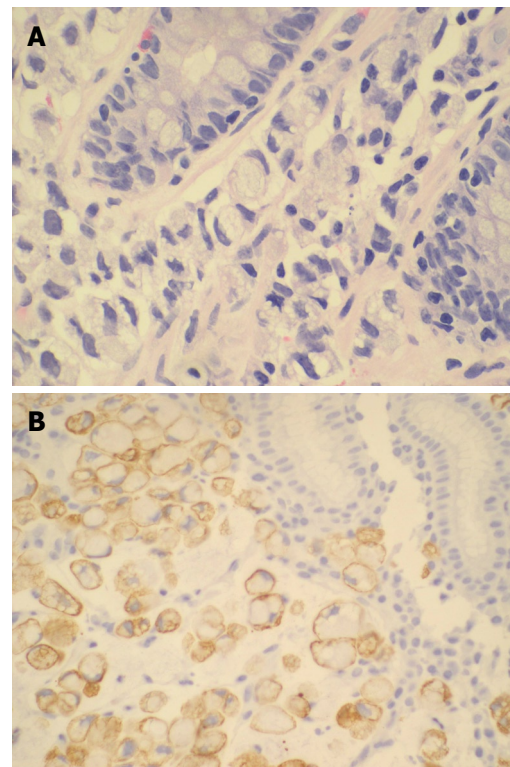


Figure 2 Photomicrograph A: normal colonic glands with signet ring cells within the lamina propria (HE); B: gastric mucosa with similar signet ring cells which were positive for CK 7.

is worse than that of colorectal adenocarcinoma and most patients present at an advanced stage. It is important to identify disseminated peritoneal disease at the time of the initial diagnosis as these patients are unlikely to benefit from surgery, if the symptoms are minimal. It is important to obtain biopsy of lesions other than the primary tumour (as was done in this case) as it may provide diagnosis of disseminated peritoneal disease.

The histological appearance of the tumour is characterized by cells with abundant intracytoplasmic mucin, which pushes the nucleus to the periphery. The tumour cells may be arranged individually or in loose clusters, and may spread diffusely through the bowel wall. Mucin lakes containing small, primitive and abortive gland structures may be present^[2,8].

Given the rarity of colon signet ring cell adenocarcinomas, the question of a primary colon or metastatic gastric adenocarcinoma frequently arises when signet ring cell carcinoma is seen on colonoscopic biopsy.

Immunostaining profiles for CK7 and CK20 have been used to characterize and differentiate signet ring cell carcinomas of breast, stomach and colon^[9,10]. CK20 is a low molecular weight cytokeratin that is normally expressed in the gastrointestinal epithelium, urothelium and in Merkel's cells^[11]. CK7 is expressed by tumours of the lung, ovary, endometrium and breast, but not of the lower gastrointestinal tract.

It has been suggested that when a signet ring cell adenocarcinoma is encountered on colon biopsy, the diagnosis of a colon primary is supported by the presence of CK7(-)/CK20(+) staining pattern in the neoplastic

cells, while gastric primary is diagnosed if the cells have a CK7(+)/CK20(-) staining pattern.

However, in the present case, the colonic specimen was positive for both CK7 and CK20, making it difficult to determine whether the primary was gastric or colonic in origin.

To date, a primary rectal signet ring cell carcinoma with peritoneal dissemination and gastric secondaries had not been described. The present case illustrates the aggressive nature of signet ring cell carcinoma and the fact that immunohistochemistry may not be 100% accurate in predicting the primary source of the signet ring cell carcinoma.

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Diagnosis and treatment of Gardner syndrome with gastric polyposis: A case report and review of the literature

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Abstract

Gardner syndrome (GS) is an autosomal dominant disease characterized by the presence of colonic polyposis, osteoma and soft tissue tumors. It is regarded as a clinical subgroup of familial adenomatous polyposis (FAP) and may present at any age from 2 mo to 70 years with a variety of symptoms, either colonic or extracolonic. We present a case of a 23-year-old female patient with GS who presented with gastric polyposis and was successively treated with restorative proctocolectomy in combination with ileal pouch anal anastomosis (RPC/IPAA), ileostomy, ileostomy closure operation, snare polypectomy during 8 mo. After operation, the patient took oral traditional Chinese medicine pills made of *Fructus mume* and *Bombyx batryticatu* for about 6 mo. The innutrition and anaemia of this patient were gradually improved. Gastroscopy showed that the remnant gastric polypi gradually decreased and finally disappeared 19 mo after the first operation. The patient had 2-3 times of solid stool per day at the time we wrote this paper.

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Key words: Gardner syndrome; Familial adenomatous polyposis; Colectomy; Ileal pouch anal anastomosis; Stomach polyposis; Hereditary nonpolyposis colorectal cancer

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INTRODUCTION

Gardner syndrome (GS) is a rare autosomal dominant inherited disorder with a high degree of penetrance characterized by intestinal polyposis, bone and soft-tissue tumours, including osteoma, epidermal inclusion cyst, lipoma, fibroma, gastric and duodenal polyposis, desmoid fibromatosis. It is regarded as a clinical subgroup of familial adenomatous polyposis (FAP). Since GC was first reported by Gardner and his colleague in early of 1950s, the association of hereditary colonic polyposis and osteomatosis with multiple cutaneous and subcutaneous tumours in GS has been extensively studied^[1-3].

Compared with FAP, the extracolonic manifestations of GC may be explained by the variable penetrance of a common mutation. The disorder is linked to the binding of 5q21-q22 (the adenomatous polyposis coli locus, *APC* gene). More than 1400 different mutations of this gene have been identified^[4]. The mutated specific area of the *APC* gene determines the extracolonic manifestations as well as the number, time frame and malignant potential of adenomatous polyps. It was reported that the mutation of *MYH* gene (1p34.3-p32.1) and environmental factors, such as diet, exercise and smoking, also play an important role in the pathogenesis of GS^[5]. Although most GS cases show familial clustering, one-third of cases occur due to spontaneous mutations.

The clinical presentation of GS is variable and its diagnosis is often delayed, despite the presence of clues for a significant amount of time. Since GS may involve different organs, it is usually very difficult to treat it, and the therapeutical effect is also uncertain. We present a rare case of a 23-year-old girl with GS who underwent a series of operations and medications which achieved satisfactory therapeutic effects.

CASE REPORT

A 23-year-old female presented with nausea, vomiting and mucous diarrhoea, occasionally with blood in the stool, for 1 mo. Colonoscopy revealed numerous polyps covering the entire colon and rectum, mostly sigmoid colon and rectum, which were consistent with the diagnosis of FAP

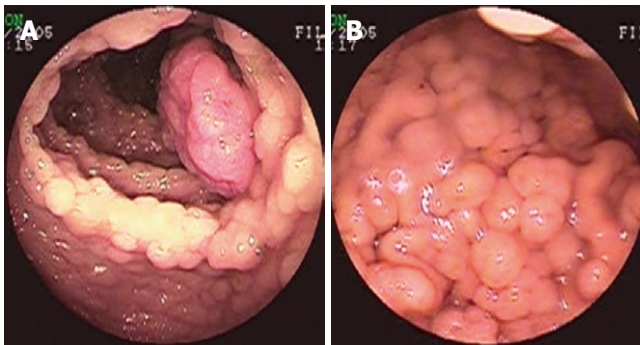


Figure 1 Preoperative endoscopy examination. **A:** Colonoscopy showing multiple polyps covering the entire colon and rectum; **B:** Gastroscopy showing multiple polyps covering the fundus and corpus ventriculi.

(Figure 1A). Gastroscopy showed numerous polyps covering the fundus and corpus ventriculi, mostly fundus ventriculi (Figure 1B). Biopsy of colon and stomach polyps showed moderate differentiation. Hemoglobin was 72 g/L. The patient was finally diagnosed as GS with innutrition and anemia.

This patient was first treated with restorative proctocolectomy in combination with ileal pouch anal anastomosis (RPC/IPAA) and ileostomy. Pathological examination of the specimen showed that the colorectal polyps were tubular adenomas with moderate differentiation. Then, an ileostomy closure operation and two times of snare polypectomy were performed. After operation, the patient took oral traditional Chinese medicine pills twice per day for about 6 mo. The traditional Chinese medicine pills were made of 3.0 g Fructus mume (smoked plum) and 3.0 g Bombyx batryticatu (stiff silkworm). Innutrition and anaemia gradually recovered. Gastroscopy showed that the remnant gastric polyps gradually decreased and finally disappeared (Figure 2A and B). The patient had 2-3 times of solid stool per day at the time when we wrote this report.

DISCUSSION

GS is considered a variant of FAP with certain extracolonic manifestations (such as osteoma, gastric or duodenal polyposis and desmoid fibromatosis). It was reported that GS is caused by truncating mutations of the *APC* gene (codons 1403 and 1578) differing from classic FAP (codons 169-1600), attenuated FAP (amino terminal to codon 157), and congenital hypertrophy of the retinal pigmented epithelium (codons 463-1387)^[4]. However, there is evidence that patients with identical mutations may have different phenotypic expressions because of unclear reasons. The majority of GS patients may have a family history, but about 25% of GS patients can present with a new dominant mutation and are the first affected member of the family. These patients are generally not under medical surveillance before they have bowel symptoms, and 67% of them may have developed colorectal cancer. In 100% of all untreated patients, cancer develops in the large intestine before the age of 40 years. Hence, prophylactic colectomy is indicated, although desmoid tumors of the

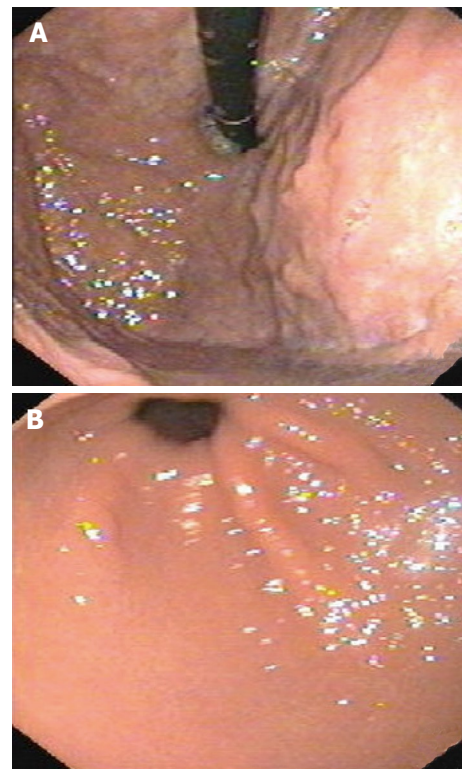


Figure 2 Post-operative gastroscopy examination shows disappearance of polyps of the fundus and corpus ventriculi (**A**) and smooth mucous membrane of the corpus ventriculi and pars pylorica (**B**).

mesenteric and abdominal wall may develop after surgery. We think that RPC/IPAA is the best operation for FAP (including GS) patients because it not only resects all large intestine mucous membranes to avoid carcinogenesis, but also preserves the intestine function and sex ability and avoids colostomy, thus improving the life quality of patients. In order to insure the concrescence of ileal pouch anal anastomosis, temporary ileostomy is occasionally necessary. After 1 to 3 mo when the anastomosis reaches its concrescence, we can perform an ileostomy closure operation. The distal ileum would play the role of colon in absorbing water from dejecta if the colon is resected, so we should take care to preserve the distal ileum for self-control defecation. The patient were underwent to a series of operations with satisfactory therapeutic effects, showing that such operations can cure similar patients.

It was reported that the carcinogenesis of gastric polyps is obviously lower than that of colorectal polyps and its carcinogenesis time may be about 10 years later than colorectal polyps^[2]. The gastric polyps in this patient gradually decreased and finally disappeared after two times of snare polypectomy and treatment with traditional Chinese medicine. Although the idiographic reason remains unclear, the prognosis of gastric polyps is well, suggesting that excessive radical operation cannot be performed for gastric polyps. It was reported that non-steroidal anti-inflammatory drugs (NSAID) can maintain the regression of colorectal adenoma in patients with FAP and hereditary nonpolyposis colorectal cancer (HNPCC)^[6-11]. Use of NSAID may pave a new secure path for the treatment FAP (including GS) and HNPCC. The component of traditional Chinese medicine is complex, the correlation of between traditional Chinese medicine and disappearance of gastric polyps is unclear. However,

reports showed that there are many organic acids and other helpful components in *Fructus mume* and *Bombyx batryticatus*, such as oxalic acid (OA), tartaric acid (TA), malic acid (MA), vitamin C (VC), lactic acid (LA), acetic acid (AA), citric acid (CA), succinic acid (SA), β -sitosterol, uracil, meso-erythritol and palmitic acid, *etc*^[12,13]. More studies are needed to make sure whether these organic acids and helpful components have the effects of NSAID.

It was reported that about 25% of patients with GS have no family history and the miniaturization of family also makes the hereditary behavior unobvious^[14-16]. It is very easy to misdiagnose these patients who have no obvious family history or colorectal polypi. We should examine the stomach, thyroid, tooth, skull and eyeground of such patients with colorectal polypi. The examination of *APC* and *MYH* mutation is helpful to differentiate patients with GS and FAP, but *APC* and *MYH* mutation examination is uncertain, thus not widely applied.

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BOOK REVIEW

Rome III: The functional gastrointestinal disorders, third edition, 2006

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Abstract

Functional gastrointestinal disorders (FGIDs) represent a common and important class of disorders within gastroenterology. Rome I, the first edition was published in 1994, with symptom-based diagnostic criteria for FGIDs. These criteria began to change the diagnostic approach to FGIDs, and no longer considered "diagnoses of exclusion" but rather "diagnoses of inclusion". Rome II, the second edition published in 2000, resulted from the continual process of analyzing new scientific and clinical evidence in the study of FGIDs. Rome II, diagnostic criteria for irritable bowel syndrome (IBS), was extended with a focus on the frequency of symptoms occurring twelve weeks (not necessarily consecutive weeks) within twelve months. ROME III, the third edition, conservative one, was published in September 2006, with changes made only where there is good evidence to do so. Some of the differences between Rome II and Rome III criteria are highlighted in this issue.

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Key words: Rome III; Functional gastrointestinal disorders; Diagnosis; Classification

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INTRODUCTION

Functional gastrointestinal disorders (FGIDs) represent

a common and important class of disorders within gastroenterology. They are a group of disorders in clinical medicine that have often posed immense problems for patients to experience, for clinicians to diagnose and treat, and for researchers to study.

The "road to Rome" began in Rome, Italy, in 1988 during the 12th International Congress of Gastroenterology, during which a working team was set up to create guidelines for the management and study of irritable bowel syndrome (IBS). After a publication outlining the classification system in 1990, several committees convened in Rome, Italy, throughout 1994 and began a process of review and analysis of the medical literature to improve the methodology for studying, diagnosing and treating 21 FGIDs. The ultimate goal was to improve the lives of patients and their families. The process has matured through three generations, producing a series of publications (Rome I, II and III), with an increased evidence-based approach to the recommendations.

ROME I AND ROME II

Rome I, the first edition published in 1994, is a compilation of documents previously published in *Gastroenterology International* over a period of 5 years by 30 international investigators who categorized the FGIDs from the esophagus to the anus. The most striking result of this process is the creation of the Rome I symptom-based diagnostic criteria for FGIDs. These criteria began to change the diagnostic approach to FGIDs, and no longer considered "diagnoses of exclusion" but rather "diagnoses of inclusion". The Rome criteria have enabled positive diagnoses without the need for extensive and unnecessary diagnostic studies to "rule out organic diseases".

Rome II, the second edition, published in 2000, resulted from the continual process of analyzing new scientific and clinical evidence in the study of FGIDs. Rome II, diagnostic criteria for irritable bowel syndrome (IBS), is extended with a focus on the frequency of symptoms occurring twelve weeks (not necessarily consecutive weeks) within twelve months.

For the first time, pediatric FGIDs were categorized, and chapters highlighting physiology of motility, sensation, brain-gut interactions, and psychosocial aspects were included.

ROME III

ROME III, the third edition, published in September

2006, is a 1048-page document written by a collaborative effort of 82 international experts. The book consists of seventeen chapters that contain the most recent information on the epidemiology, pathophysiology, diagnosis, and treatment of FGIDs. Diagnostic criteria for some of the FGIDs have been revised. "Red flag" symptoms and signs that warrant further diagnostic evaluation have been included. Suggestions for when to make a mental health referral have also been given with new chapters on pharmacology and pharmacokinetics, sociocultural perspectives related to gender, age, and cultural impact. One chapter is also devoted to the development and validation of the Rome III: Diagnostic Questionnaire. New appendices contain validated Rome III: adult and pediatric questionnaires and a table comparing Rome II and Rome III diagnostic criteria.

CHANGES IN ROME III

The Rome III process is a conservative one, with changes made only where there is good evidence to do so. The following is a summary of the changes in criteria and other recommendations along with their justification.

Time frame change for FGIDs

The time frame for a diagnosis now originates at 6 mo prior to clinical presentation and diagnosis and must be currently active (i.e., meet criteria) for 3 mo. This time frame is less restrictive than that in Rome II (12 wk of symptoms over 12 mo) and is easier to understand in a questionnaire or for research and clinical practice.

Changes in classification categories

The Rome II: category of Childhood Functional GI Disorders, is now classified as Childhood Functional GI Disorders: Neonate/Toddler (Category G) and Childhood Functional GI Disorders: Child/Adolescent (Category H). This reflects the different clinical conditions existing between the two categories relating to the growth and development of the child, thus removing Functional abdominal pain syndrome (FAPS) from functional bowel disorders (Category C) into its own category (Category D). This is based on the growing evidence that FAPS relates more to CNS amplification of normal regulatory visceral signals rather than functional abnormalities *per se* within the GI tract. The committee members selected for this new category included psychologists, psychiatrists and gastroenterologists involved in brain gut interactions.

Criteria changes

In Rome III, functional dyspepsia is de-emphasized as an entity for research, due to its symptom heterogeneity. Instead, the gastroduodenal committee has recommended using an umbrella term "dyspepsia symptom complex" which is subclassified into two conditions that may overlap: (1) Postprandial Distress Syndrome, and (2) Epigastric Pain Syndrome. Although similar to the

dysmotility like and ulcer like dyspepsia in Rome II, there now are several items for the criteria derived from factor analytic studies and physiological support instead of being based on the single symptom of epigastric discomfort or pain. Further studies are needed to validate this change. Functional biliary tract disorders have been a challenging group of disorders to diagnose and treat. These disorders are of low prevalence in comparison to other FGIDs, but they tend to be investigated with invasive and risky studies, such as endoscopic retrograde pancreatography (ERCP) or sphincter of Oddi manometry, and treated with unnecessary endoscopic sphincterotomy and surgery. Rome III recommends more restrictive evaluation of these disorders.

Rome III is the single most comprehensive and authoritative resource on the subject of FGIDs. It is readable, well organized, clearly labeled, and extensively referenced. The 82 experts who participated in the Rome III process have created an outstanding work from which clinicians, clinical investigators, basic scientists, mental health providers, the pharmaceutical industry and, most of all, patients with FGIDs will greatly benefit.

CONCLUSION

The information obtained in Rome III is comprehensive although certainly not complete. It is likely that the next few years will bring considerable advances in our understanding and treatment of these disorders, and when that occurs, revision of such information should be planned before moving to Rome IV.

Brain imaging, using positron emission tomography, functional magnetic resonance imaging, or other modalities^[1-3] (Drossman 2005, Hobson 2004, and Hobson 2005) provides an opportunity to assess brain function in response to visceral stimulation^[4,5] (Kern 2002, Yaguez 2005) among healthy subjects and patients with FGIDs. Developing standardization for brain imaging assessment and making recommendations relating to symptom severity for research and clinical care should be emphasized in the next edition.

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LETTERS TO THE EDITOR

Role of *ABCC2* common variants in intrahepatic cholestasis of pregnancy

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Abstract

The pathogenesis of intrahepatic cholestasis of pregnancy (ICP), a disorder that adversely affects maternal wellbeing and fetal outcome, is unclear. However, multiple factors probably interact along with a genetic predisposition. We would like to add some comments on a paper recently published concerning the role of *ABCB11* and *ABCC2* polymorphisms in both ICP and contraceptive-induced cholestasis, especially in the light of our recently published findings about a positive association between ICP and *ABCC2* common variants.

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Key words: Intrahepatic cholestasis of pregnancy; *ABCC2*; MRP2; Gene variants

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TO THE EDITOR

We read with great interest the article of Meier *et al.*

about the role of *ABCB11* and *ABCC2* polymorphisms in both intrahepatic cholestasis of pregnancy (ICP) and contraceptive-induced cholestasis^[1]. The authors observed an association between the *ABCB11* 1331T > C polymorphism and the above mentioned cholestatic disorders. Additionally, the authors claimed of a lack of association with 3 single nucleotide polymorphisms (SNPs) in *ABCC2* gene, concluding that common *ABCC2* polymorphisms are not associated with the development of ICP.

We would like to make several comments on these findings, particularly concerning to the lack of association with *ABCC2* gene variants. First of all, a note of caution should be added to the reported findings, as the markers assumed by the authors as common variants (rs2273697, rs17222723 and rs8187710), except for rs2273697, include one allele at a very low frequency, as the rs17222723-A allele frequency is 0.067 and the rs8187710-A allele frequency is 0.059, at least for data from the HapMap project for Caucasians and from a rough estimation of own author's data^[1]. Consequently, in the group of patients included by the authors only 5 out 33 patients showed either the rs172227234 or rs8187710 heterozygous AT or AG genotype, respectively, and none of them showed the homozygous AA genotype. The same happened in the pregnant control group. Thus, in this frame, the statistical power of the study even taking into account the additive genetic model is very low (less than 20%) owing to the lower MAF of these variants.

Second, we wish to note that we recently published^[2] a candidate gene association study showing the contribution of six *ABCC2* gene variants to the risk of ICP. The study involved promoter, coding and non-coding regions of *ABCC2* (4 tag SNPs representing 46 polymorphic sites located in 70 kb of the gene, in addition to the rs17222723 and rs8187710), and showed that, at least, one of the *ABCC2* variants (rs3740066) at the exon 28 was significantly associated with ICP with its estimated risk of disease for homozygous AA subjects being 4-fold higher than that for homozygous GG subjects (OR, 4.44; 95% CI, 1.83-10.78; *P* < 0.001). Although more studies are necessary to establish whether rs3740066 is the causal variant or one linked to it, our results suggest that ICP may be associated with the common variants of *ABCC2*. Interestingly, we also included in the analysis rs172227234 and rs8187710, and we did not observe significant differences in genotype frequencies of the 2 SNPs in ICP and controls.

In conclusion, we consider that the pathogenic involvement of *ABCC2* (*MRP2*) in ICP is still an open issue, particularly in the frame of a small number of studies about the role of the *ABCC2* gene variants in cholestatic disorders, with the exception of the Dubin Johnson phenotype.

Although the major physiological function of *ABCC2* is to transport conjugated metabolites into the bile canaliculus, previous data demonstrated that a major metabolite of human estrogen metabolism, estradiol-17- β -D-glucuronide (E217 β G), has been shown to be transported by both *MRP2* and *MRP3*^[3]. These findings support that *ABCC2* represents an alternative candidate protein involved in the pathogenesis of hormonal cholestasis. Additionally, it has been shown that *MRP2* is regulated by three distinct nuclear receptor signaling pathways that converge on a common response element in the 5'-flanking region of this gene^[4]. Hence, the intimate mechanism, by which gene variants including *ABCC2* may influence ICP susceptibility, is not fully explored and a complex network involving nuclear receptors and other transcription factors may be the cause of liver injury in cholestatic disorders^[5].

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Meetings

Events Calendar 2008-2009

FALK SYMPOSIA 2008

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

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

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

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

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

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

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

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

April 05-09, Monte-Carlo (Grimaldi Forum), Monaco
 OESO 9th World Congress, The Gastro-esophageal Reflux Disease: from Reflux to Mucosal Inflammation - Management of Adeno- carcinomas
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April 18-22, Buenos Aires, Argentina
 9th World Congress of the International Hepato-Pancreato Biliary Association
 Association for the Study of the Liver
www.ca-ihpba.com.ar

April 23-27, Milan, Italy
 43rd Annual Meeting of the European

Association for the Study of the Liver
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Value of colonoscopy for prediction of prognosis in patients with ulcerative colitis

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Abstract

Ulcerative colitis (UC) is a chronic inflammatory bowel disorder characterized by exacerbations and remissions. Some UC patients remain refractory to conventional medical treatment while, in others, the effectiveness of drugs is limited by side-effects. Recently, cyclosporine and leukocyte removal therapy have been used for refractory UC patients. To predict the efficacy of these therapies is important for appropriate selection of treatment options and for preparation for colectomy. Endoscopy is the cornerstone for diagnosis and evaluation of UC. Endoscopic parameters in patients with severe or refractory UC may predict a clinical response to therapies, such as cyclosporine or leukocyte removal therapy. As for the patients with quiescent UC, relapse of UC is difficult to predict by routine colonoscopy. Even when routine colonoscopy suggests remission and a normal mucosal appearance, microscopic abnormalities may persist and relapse may occur later. To more accurately identify disease activity and to predict exacerbations in UC patients with clinically inactive disease is important for deciding whether medical treatment should be maintained. Magnifying colonoscopy is useful for the evaluation of disease activity and for predicting relapse in patients with UC.

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Key words: Ulcerative colitis; Colonoscopy; Prediction of outcome

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INTRODUCTION

Ulcerative colitis (UC) is a chronic inflammatory bowel disorder characterized by diffuse mucosal inflammation of the colorectum with exacerbations and remissions^[1-5]. Approximately 15% of patients experience a severe exacerbation requiring hospital admission at some time during their illness^[3,6]. The purpose of treatments for patients with ulcerative colitis is achieving remission and maintaining quiescence of the disease. Patients with UC must rely on multiple medications to control their symptoms, including aminosaliclates, corticosteroids and purine analogs. Although decades of clinical experience in the management of UC have allowed the optimization of approaches to the induction and maintenance of remission, some patients remain refractory to conventional medical treatment and the effectiveness of these drugs may be limited by side-effects^[7-11]. The use of immunosuppressive agents, including purine analogs, now constitutes a therapeutic modality for the treatment of UC^[12]. Although highly effective, a disadvantage of these drugs is the significant delay in their onset of clinical benefit, which limits their utility to the treatment of severe disease.

Although the degree of inflammation as assessed by routine colonoscopy is a reliable parameter of disease activity, discrepancies between colonoscopic appearance and histopathologic abnormalities are sometimes seen in patients with clinically inactive UC (Figure 1). Even when routine colonoscopy suggests remission and a normal mucosal appearance, microscopic abnormalities may persist^[13,14] and relapse may occur later^[15]. A recently developed high-resolution video-magnifying colonoscope has enabled the observation of pit patterns on the

surface of the colorectal mucosa. This in turn allows an understanding of the morphological relationship between the pit patterns detected colonoscopically and the crypts observed histopathologically^[16-20]. As far back as 1980, Poulsen *et al*^[21] examined biopsy specimens from the rectal mucosa of UC patients under a stereomicroscope and found microstructural abnormalities in the mucosal surface in almost every patient, as well as a close correlation between stereomicroscopic features and the clinical disease activity, sigmoidoscopic findings, and histologic activity of the disease.

Here, we discuss endoscopic factors predictive of the efficacy of therapies in patients with intractable UC, and endoscopic factors that may predict the probability of subsequent disease relapse in UC patients in remission. We will reconsider the value of endoscopy when we treat UC patients.

ENDOSCOPIC PREDICTORS OF RESPONSE TO THERAPIES IN PATIENTS WITH REFRACTORY ULCERATIVE COLITIS

In recent years, steroid-refractory cases of UC have been successfully treated by adding intravenous cyclosporine to the glucocorticosteroids. Cyclosporine is a lipophilic cyclic peptide that interrupts the cellular immune response by blocking interleukin 2 productions by T cells. Uncontrolled studies show that approximately 80% of patients with severe UC refractory to glucocorticosteroid treatment respond to cyclosporine therapy^[22,23]. The use of cyclosporine is, however, associated with considerable morbidity. Serious complications such as *Pneumocystis carinii* pneumonia and seizures have occurred in as many as 12% of patients in large series, and deaths have been reported^[24-26]. Less serious, but nevertheless troubling, side-effects including hypertension, liver and renal impairment, tremor, paresthesia and headache, occur in up to 50% of patients^[23,25-27]. It would be useful to define factors predictive of response to cyclosporine treatment for severe flares of ulcerative colitis, to avoid the side effects as well as reduce the risk of subjecting the patients to increased morbidity and mortality due to needlessly delaying colectomy. However, there has been only limited information as to which factors are associated with a response to cyclosporine that leads to possible avoidance of colectomy in such patients. Rowe *et al* demonstrated that a higher percentage of band neutrophils on admission was predictive of patients who were unlikely to respond to cyclosporine and who would require colectomy^[28]. On the other hand, McCormack *et al* showed that the *in vitro* cyclosporine sensitivity of proliferating lymphocytes was predictive of the therapeutic response^[29]. Genetic factors of the host are also considered to play a role in UC outcomes. The TT genotype of exon 21 multidrug resistance gene 1 polymorphisms is associated with a higher risk of cyclosporine failure in patients with steroid resistant UC^[30]. Our prospective analysis with a logistic regression model, colonoscopic findings predictive of response to intravenous cyclosporine in patients with

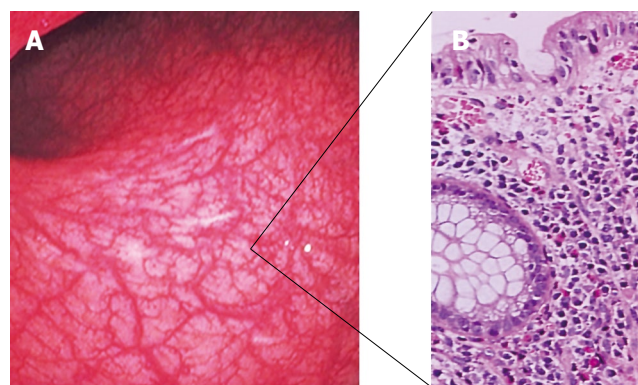


Figure 1 A case of inactive UC. A discrepancy is seen between an endoscopic and a histologic finding. **A:** A routine colonoscopy finding. It shows an almost normal mucosal appearance; **B:** A histologic finding. It shows an intense infiltration of mononuclear cells and neutrophils.

Table 1 Colonoscopic finding predictive of response to intravenous cyclosporine in ulcerative colitis patients

	Responders (n = 17)	Non-responders (n = 9)	Relative risk ¹ (Odds ratio)
Deep and extensive ulcerations (yes:no)	8:9	0:9	14.20 (P < 0.005)
Mucosal bleeding (yes:no)	5:12	7:2	0.12 (P < 0.05)
Poor luminal extensibility (yes:no)	4:13	7:2	0.09 (P < 0.01)

¹Logistic regression analysis.

steroid-resistant ulcerative colitis included the presence of deep and extensive ulcerations, and the absence of mucosal bleeding or poor luminal extensibility (Table 1).

Findings in active UC include the activation and extravasation of large numbers of granulocytes and monocytes/macrophages into the colonic mucosa^[31,32]. These infiltrated leukocytes may cause extensive mucosal tissue injury by releasing degradative proteases^[32-34], reactive oxygen derivatives^[32,34,35], and pro-inflammatory cytokines^[36]. Leukocyte removal therapy is recognized as a second novel strategy for the treatment of steroid-refractory UC, based on the assumption that this non-drug therapy attenuates intestinal inflammation by reducing excess and activated granulocytes, monocytes and lymphocytes from the circulating blood before they reach the inflamed mucosa^[37]. Adsorption with beads (granulocytapheresis, GCAP) or filters (leukocytapheresis, LCAP) is most commonly used^[38,39]. Several studies have reported the beneficial effects of leukocyte removal therapy on both the induction and maintenance of clinical remission in patients with IBD^[40-42], suggesting that it may be a useful adjunct to conventional therapy in patients with active severe UC and those refractory to conventional drugs. Further, leukocyte removal therapy might be an effective first line medication^[43]. First UC episode and short disease duration are good predictors of response to leukocyte removal therapy^[44]. Steroid-naïve patients respond particularly well to this treatment^[42,45]. Patients with deep colonic lesions might be less satisfactory^[45]. However, our prospective analysis in patients

with steroid resistant ulcerative colitis did not find any colonoscopic findings predictive of response to leukocyte removal therapy^[46]. Further study with a larger population of patients needs to be conducted to define predictors of response to cyclosporine or leukocyte removal therapy, including prolonged outcome, for more appropriate selection of treatment options with these therapies in patients with severe ulcerative colitis.

PREDICTION OF RELAPSE IN PATIENTS WITH QUIESCENT ULCERATIVE COLITIS

Severity in ulcerative colitis is generally assessed using symptoms, laboratory data^[47], colonoscopic findings^[48-55] and histologic degree of inflammation in the biopsy specimens^[15,56-59]. Histopathologic assessment is considered the standard for evaluation of disease activity in patients with ulcerative colitis^[60]. The observation under conventional colonoscopy has been regarded as useful for the evaluation of disease activity, since it offers direct observation of mucosal changes, but it still remains controversial whether colonoscopic grade correlates with histopathologic findings. It has been reported that the degree of histologic inflammation within biopsy specimens did not necessarily correlate with endoscopic abnormalities^[48,49,61,62]. It is not unusual for routine colonoscopy performed to assess the stage of UC to show quiescent colitis despite the histological persistence of inflammation^[48,61,63], which later results in the relapse of colonic inflammation^[15]. Hurlstone DP *et al* reported high-frequency ultrasound is a valid adjunctive 'tool' for the trans-mural assessment of the colorectal wall in UC^[64]. This technique may aid in the initial diagnosis, and ongoing chronic management of disease.

Matsumoto *et al* reported usefulness of magnifying chromoscopy for the assessment of severity in UC patients^[65]. In their study, magnifying colonoscopy was performed in 41 patients with ulcerative colitis, and the findings in the rectum were graded according to network pattern (NWP) and cryptal opening (CO). The clinical, endoscopic and histologic grades of activity were not different between groups divided by the presence or absence of each finding. However, when the two features were coupled, patients with visible NWP and CO had a lower clinical activity index and lower grade of histologic inflammation than those in whom both findings could not be visualized. Furthermore it has been suggested that the presence of branches in surface epithelium may be a factor that predicts future disease relapse^[15], and they suggested that altered pattern as defined by magnified colonoscopic views may be predictive of the course of ulcerative colitis^[65].

Fujiya *et al* proposed the classification of magnifying colonoscopic findings in patients with ulcerative colitis which is useful for the evaluation of disease activity and for the prediction of periods of remission^[66]. The classification was devised as follows: regularly arranged crypt opening, villous-like appearance, minute defects of epithelium (MDE), small yellowish spots (YS), and coral reef-like appearance. The colonoscopic findings by classification

were compared with histopathologic findings in 61 patients and the usefulness of the classification for predicting relapse was prospectively analyzed in 18 patients. Under conventional colonoscopic examinations, all areas evaluated as Matts grade 1 had a corresponding histopathologic grade 1. In contrast, most areas assessed as Matts grade 3 or 4 were diagnosed as histopathologic grade 3 or higher. However, grade 2 mucosa had histopathologic findings that varied from quiescent to active disease. These suggest that normal and diseased mucosas are easily recognized by conventional colonoscopy, but it is difficult for conventional colonoscopy to assess the minute mucosal changes that reflect smoldering histopathologic inflammation^[48,49,61]. In contrast, under magnifying colonoscopic examinations, 37 (82.2%) of the 45 areas in which regularly arranged crypt openings or a villous-like appearance was detected had a corresponding histopathologic grade 1, and all areas in which MDE, SYS, or the coral reef-like appearance was observed had a corresponding histopathologic grade 2 or higher. In this study, the correlation between histopathologic grade and magnifying colonoscopic findings ($r^2 = 0.807$) was better than that for histopathologic grade versus conventional colonoscopy ($r^2 = 0.665$). This study found that patients in whom MDE was observed during clinical remission frequently had a relapse within short periods (6 mo) compared with patients without these findings, and 50% of patients who underwent clinical remission still had active inflamed mucosa with MDE, which correlates with the results of previous studies in which 30% to 60% of patients in remission, as determined by clinical symptoms, were still in the active stage of ulcerative colitis based on histopathologic findings^[49,62]. In our study we found that magnifying colonoscopy (MCS) grade was associated with the degree of histological inflammation in quiescent patients with ulcerative colitis, and might predict the probability of subsequent disease relapse in patients with ulcerative colitis in remission. Magnifying colonoscopy was performed in 112 patients with ulcerative colitis in remission. The relationship between pit patterns and histological disease activity was evaluated. Pit patterns in the rectal mucosa were classified into three MCS grades on the basis of size, shape, and arrangement (Figure 2). The patients were followed until relapse or a maximum of 12 mo. A positive correlation was identified between MCS grade and histological grade (Figure 3). Multivariate proportional hazard model analysis showed that MCS grade was a significant predictor of relapse. Kaplan-Meier estimate of relapse during 12 mo' follow up was found to increase with increasing MCS grade, with a percentage of 0 for grade 1, 19% for grade 2, and 43% for grade 3 (Figure 4). Although MCS grade positively correlated with histological grade, histological grade was less accurate predictors of disease relapse. One reason may be that they are assessed in biopsy specimens derived from a specific and limited area, whereas magnifying colonoscopy allows the observation of a more extended and representative area of colorectal mucosa, and accordingly greater accuracy by MCS grading.

CONCLUSION

Endoscopic parameters in patients with severe or refractory

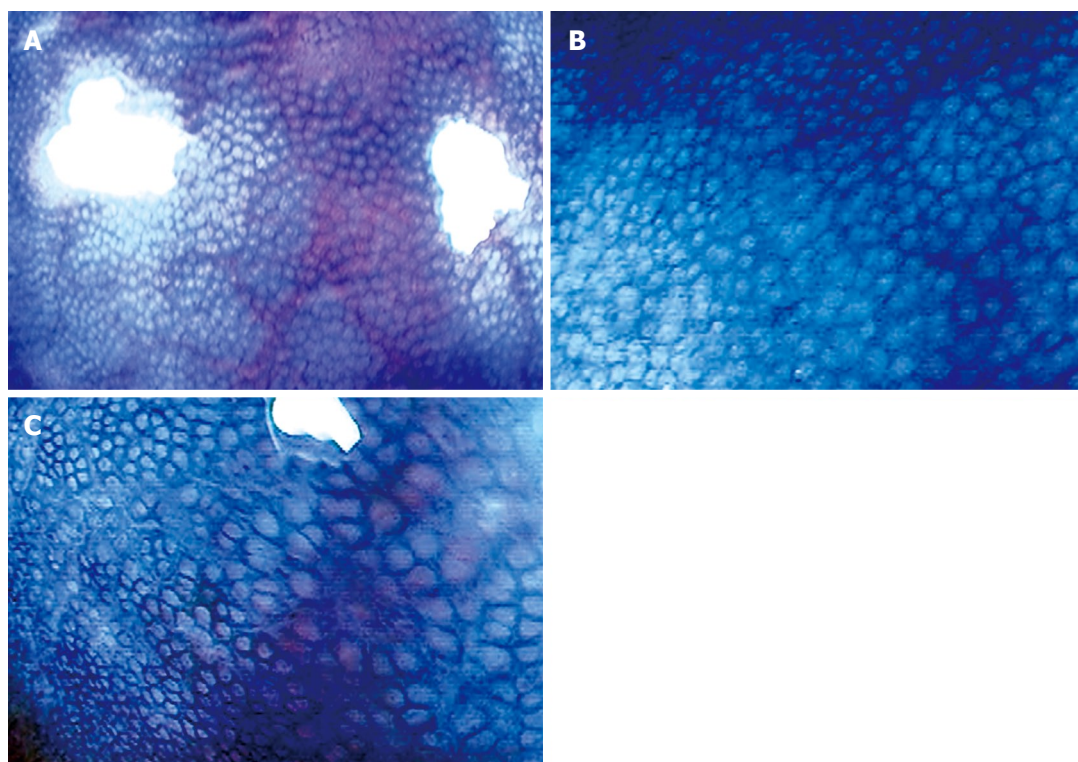


Figure 2 Grading of pit structures in the colorectal mucosa of patients with inactive UC. **A:** MCS grade 1, pits small, round, and regularly arranged; **B:** MCS grade 2, pits rather large, oval, and somewhat irregular in arrangement; **C:** MCS grade 3, pits of various shapes and sizes, and irregularly arranged.

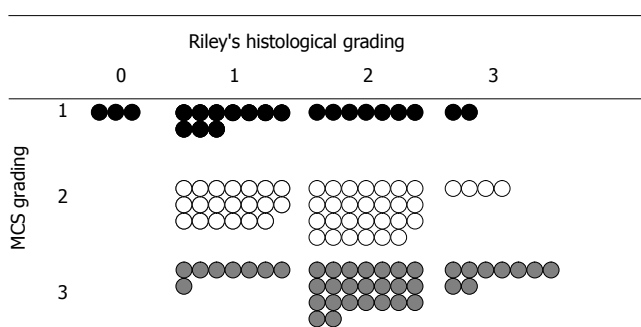


Figure 3 Correlation between grading of pit structures and histological findings ($P < 0.001$, Spearman's rank test). The number of colored ring indicates the number of the patients performed magnifying colonoscopy ($n = 112$).

UC may predict a clinical response of the therapies, such as cyclosporine or leukocyte removal therapy. Magnifying colonoscopy is useful for the evaluation of disease activity and for predicting relapse in patients with ulcerative colitis. Endoscopy is the cornerstone for diagnosis and evaluation of UC, and advanced imaging techniques, including chromoendoscopy, magnification endoscopy, confocal endomicroscopy, and spectroscopy, may aid in this field. Further studies remain to be done to define endoscopic predictors of response to therapy or outcome of UC patients.

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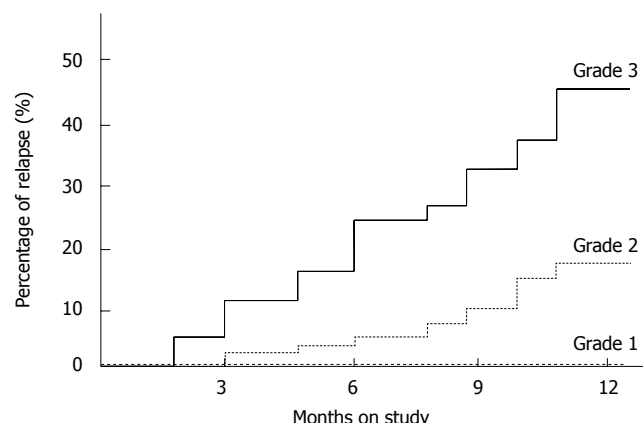


Figure 4 Kaplan-Meier survival analysis showing the cumulative proportion of patients who had a relapse according to MCS grade group.

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MUC1 and colorectal cancer pathophysiology considerations

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Abstract

Several lines of evidence point towards a biological role of mucin and particularly MUC1 in colorectal cancer. A positive correlation was described between mucin secretion, proliferation, invasiveness, metastasis and bad prognosis. But, the role of MUC1 in cancer progression is still controversial and somewhat confusing. While Mukherjee and colleagues developed MUC1-specific immune therapy in a CRC model, Lillehoj and co-investigators showed recently that MUC1 inhibits cell proliferation by a β -catenin-dependent mechanism. In carcinoma cells the polarization of MUC1 is lost and the protein is over expressed at high levels over the entire cell surface. A competitive interaction between MUC1 and E-cadherin, through β -catenin binding, disrupts E-cadherin-mediated cell-cell interactions at sites of MUC1 expression. In addition, the complex of MUC1- β -catenin enters the nucleus and activates T-cell factor/leukocyte enhancing factor 1 transcription factors and activates gene expression. This mechanism may be similar to that just described for DCC and UNC5H, which induced apoptosis when not engaged with their ligand netrin, but mediate signals for proliferation, differentiation or migration when ligand bound.

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Key words: Mucin; MUC1; Glycoprotein; Colorectal cancer; Gastrointestinal oncology; Carcinogenesis; Metastasis; Tumorigenicity

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MUC1 is a structural membranous bound mucin, expressed on the apical borders of secretory epithelial cells which previously had many names such as DF3, episialin, CA5-3, PAS-O, polymorphic epithelial mucin (PEM), or epithelial membrane antigen^[1].

MUC1 gene is located on chromosome 1q21 and has 1201 nucleotides. The N-terminal ectodomain of MUC1 (MUC1-N) consists of variable numbers of 20-amino-acid tandem repeats (VNTRs), with the number of repeats varying from 20 to 120 in different individuals^[2]. These sites are subject to O-glycosylation that contributes to form a structure that extends beyond the glycocalyx of the cell. The protein has a protective function by binding to pathogens and also functions in a cell signaling capacity^[3].

MUC1-N is tethered to the cell membrane as a heterodimer with the MUC1 C-terminal subunit (MUC1-C), which includes a 58-amino acid extracellular domain, a 28-amino acid transmembrane domain, and a 72-amino acid cytoplasmic tail that contains sites for tyrosine and serine phosphorylation^[4]. Over expression, aberrant intracellular localization, and changes in glycosylation of this protein as found in most human carcinomas, confers anchorage-independent growth and tumorigenicity^[5]. Other studies have demonstrated that over expression of MUC1 confers resistance to apoptosis induced by oxidative stress and anti-cancer agents^[6]. Multiple alternatively spliced transcript variants that encode different isoforms of MUC1 have been reported, but the full-length nature of only some of them has been determined^[7].

Several lines of evidence point towards a biological role of mucin and particularly MUC1 in colorectal cancer (CRC). A positive correlation was described between mucin secretion, proliferation, invasiveness, metastasis and bad prognosis^[8-10]. When MUC1 was expressed at the deepest tumor invasive portion, lymphatic and venous invasion was more pronounced as well as lymph nodes and liver metastasis^[11]. Correlation with bad prognosis was found in mismatch repair (MMR) - proficient colorectal tumors, but not in MLH1 negative tumors or in Lynch syndrome (HNPCC)^[12].

But, the role of MUC1 in cancer progression is still controversial and somewhat confusing. While Mukherjee and colleagues, in a very sophisticated way, developed MUC1-specific immune therapy in a CRC model, Lillehoj and co-investigators showed recently that MUC1 inhibits cell proliferation by β -catenin-dependent mechanism^[13,14]. A similar observation was described by Yuan and co-workers^[15].

Interaction of the cytoplasmic tail of MUC1 with β -catenin has a significant effect on cell cycle and

proliferation. This process hardly happens in normal polarized epithelium, because MUC1 resides on the apical surface while β -catenin resides on the lateral surface. Loss of polarity during transformation creates a permissive environment for MUC1 and β -catenin interaction^[7].

β -catenin can bind directly to the amino acid sequence 50-SAGNGGSSL-59 of the MUC1 cytoplasmic domain (a similar binding site is found on E-cadherin and APC proteins). The binding is promoted by phosphorylation of T41 by Ser/Thr kinase PKC ζ and of Y46 by Src or EGFR^[16]. Inhibition of β -catenin binding to MUC1 is the result of phosphorylation of S44 by GSK3 β that can also directly degrades β -catenin. Disruption of the β -catenin binding site in MUC1 suppresses its ability to induce anchorage-dependent and independent growth, indicating β -catenin binding to MUC1 is a critical component of its tumorigenic activity. MUC1 also protects β -catenin from degradation by GSK3 β , and when co-localized with β -catenin in the nucleus co activates transcription of Wnt target genes^[17-19]. MUC1 binding to β -catenin suppresses its ability to interact with E-cadherin at adherent junctions, leading to a breakdown in cell-cell interactions. GSK3 β -mediated disruption of the complex restores the E-cadherin/ β -catenin interaction^[20].

In carcinoma cells the polarization of MUC1 is lost, and the protein is over expressed at high levels over the entire cell surface. A competitive interaction between MUC1 and E-cadherin, through β -catenin binding, disrupts E-cadherin-mediated cell-cell interactions at sites of MUC1 expression. In addition, the complex of MUC1- β -catenin enters the nucleus and activates T-cell factor/leukocyte enhancing factor 1 (Tcf/LEF-1) transcription factors and activates gene expression^[18]. This enhances proliferation, and decreases cell-cell adhesion which may both increase carcinogenesis and metastasis. GSK3 β interacts with the STDRSPYE motif in MUC1, phosphorylates the serine in this domain, and prevents binding of β -catenin^[16].

It is proposed that APC binding prevents the formation of β -catenin-Tcf complex, and that MUC1 binding prevents β -catenin- α -catenin-E-cadherin complex. GSK3 β interacts with β -catenin to restore β -catenin-E-cadherin complex, and with APC to bind β -catenin and prevent β -catenin-Tcf complex (Figure 1). The exact mechanism of MUC1 associated cancer cell proliferation and carcinogenesis is not well understood. MUC1 can bind β -catenin, prevents its entering the nucleus or activating Tcf/LEF-1, and inhibits proliferation. On the other hand, MUC1-C complex with β -catenin may enter the nucleus and the opposite action will take place. In both cases, β -catenin binding to MUC1 will prevent its binding to E-cadherin or to APC.

The cytoplasmic tail of MUC1 contains 4 tyrosine residues that are phosphorylated before binding β -catenin. It is speculated that MUC1 is a membranous receptor which maintains cell cycle progression or enhances apoptosis. Activating MUC1 will result in phosphorylation of the tyrosines on the cytoplasmic tail and binding β -catenin. This will prevent β -catenin from binding E-cadherin or activating Tcf/LEF-1 pathway. This mechanism may be similar to that just described for DCC and UNC5H, which

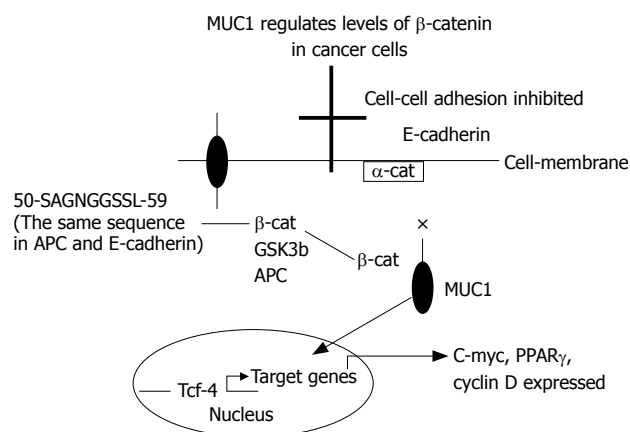


Figure 1 It is proposed that APC binding prevents the formation of β -catenin-Tcf complex, and that MUC1 binding prevents β -catenin- α -catenin-E-cadherin complex. GSK3 β interacts with β -catenin to restore β -catenin-E-cadherin complex, and with APC to bind β -catenin and prevent β -catenin-Tcf complex. \times = inhibition; arrow = enhancing.

induced apoptosis when not engaged with their ligand netrin, but mediate signals for proliferation, differentiation or migration when ligand bound^[21].

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REVIEW

Necrotizing enterocolitis: A multifactorial disease with no cure

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INTRODUCTION

Necrotizing enterocolitis (NEC) is an inflammatory bowel disease of neonates and remains one of the most common gastrointestinal emergencies in newborn infants^[1]. Onset of NEC is often within the first three months of life and neonates who are of extremely low birth weight (< 1000 g) and under 28 wk gestation are the most susceptible^[2]. Full term neonates account for 10% of all NEC cases while premature infants account for 90%^[3]. With an incidence rate of 1%-5% for all newborns admitted to the NICU^[1], a prevalence of 7%-14% of very low birth weight infants (VLBW, 500-1500 g)^[4] and a mortality rate approaching 20%-50%^[5], NEC continues to represent a significant clinical problem. In Canada, the incidence rate is 1.8 per 100 live births with a prevalence of 7% of VLBW infants^[1]. Advances in obstetric and neonatal care have improved survival rates for smaller, more immature infants, and as more VLBW preterm infants survive the neonatal period, the population at risk for NEC increases^[1]. No consistent association between sex, race, and rates of NEC has been identified. However, male VLBW infants and black infants are at greater risk of death^[6]. Due to inadequate treatments and no effective preventative strategy, an estimated 20%-40% of babies with NEC require surgery^[1] and 10%-30% experience significant morbidity including neurodevelopmental impairment, vision and hearing impairment, failure to thrive, feeding abnormalities, diarrhea, bowel obstruction, and short bowel syndrome^[1,2,7]. The case fatality rate with surgical intervention is as high as 50%^[1]. NEC is also a financial burden to the health care system with yearly hospital charges reported to be as high as \$6.5 million in the US^[8]. Thus, NEC continues to be an important health issue for preterm neonates.

DIAGNOSIS

Clinical signs and symptoms

The onset of NEC can occur suddenly within a few

Abstract

Necrotizing enterocolitis is an inflammatory bowel disease of neonates with significant morbidity and mortality in preterm infants. Due to the multifactorial nature of the disease and limitations in disease models, early diagnosis remains challenging and the pathogenesis elusive. Although preterm birth, hypoxic-ischemic events, formula feeding, and abnormal bacteria colonization are established risk factors, the role of genetics and vasoactive/inflammatory mediators is unclear. Consequently, treatments do not target the specific underlying disease processes and are symptomatic and surgically invasive. Breast-feeding is the most effective preventative measure. Recent advances in the prevention of necrotizing enterocolitis have focused on bioactive nutrients and trophic factors in human milk. Development of new disease models including the aspect of prematurity that consistently predisposes neonates to the disease with multiple risk factors will improve our understanding of the pathogenesis and lead to discovery of innovative therapeutics.

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Key words: Necrotizing enterocolitis; Diagnosis; Pathogenesis; Prevention; Disease models; Vasoactive/inflammatory mediators

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Table 1 Bell's staging criteria for necrotizing enterocolitis

Stage	Systemic signs	Intestinal signs	Radiological signs
I A (Suspected)	Temperature instability, apnea, bradycardia, lethargy	Poor feeding, emesis, ↑ pre-gavage residuals, mild abdominal distension	Normal or intestinal dilation, mild ileus
I B (Suspected)	Same as above	Above and blood from rectum	Same as above
II A (Proven)	Same as above	Above + absent bowel sounds + mild abdominal tenderness	Intestinal dilation, ileus, pneumatosis intestinalis
II B (Proven)	Above + metabolic acidosis + thrombocytopenia	Above + definite abdominal tenderness	Above + portal vein gas + possible ascites
III A (Advanced)	Above + hypotension, respiratory acidosis, neutropenia	Above + peritonitis, marked distension of abdomen	Above + definite ascites
III B (Advanced)	Same as above	Same as above	Above + pneumoperitoneum

hours or may be preceded by several days of feeding intolerance^[7]. Age at presentation is inversely related to gestational age at birth, with full-term infants often presenting in the first few days of life^[1]. NEC affects the gastrointestinal tract and, in severe cases, may have profound systemic impact^[9]. Initial symptoms may be subtle and can include feeding intolerance (gastric residuals, bilious vomiting), bloody diarrhea, temperature instability, lethargy, apnea, bradycardia, decreased peripheral perfusion, delayed gastric emptying, ileus, abdominal distension, or tenderness and respiratory stress^[1,9,10]. Non-specific laboratory abnormalities can include neutropenia, thrombocytopenia, hyponatremia, hyperglycemia, metabolic acidosis, and bacteria or infectious products isolated from blood, urine, or stool^[9,11]. Serial C-reactive protein could be useful in the management of the disease. C-reactive protein distinguishes stage I NEC from ileus or benign pneumatosis and high levels predict development of complications such as strictures, abscess, or need for surgery^[12]. Because early signs of this disease are non-specific, sepsis may be suspected before NEC^[1].

Pathological findings

The ileum and proximal colon are the most commonly affected sites in NEC although any segment of the gastrointestinal tract can be involved including the stomach^[13]. Severity of bowel wall necrosis ranges from a small localized mucosal necrosis of a bowel segment to transmural necrosis of the entire small intestine and colon in most severe cases^[7]. In more advanced stages of NEC, pathological findings include gastrointestinal bleeding, inflammation, bacterial overgrowth, intestinal distension with multiple dilated loops of small bowel, pneumatosis intestinalis and portal air, intestinal perforation, coagulative necrosis, hypotension, septic shock, pneumoperitoneum, and intraabdominal fluid^[2,10]. In 1978, Bell and colleagues^[14] proposed a system for the uniform clinical staging of infants with NEC. They classified infants as having stage I (suspect), stage II (definite), or stage III (advanced)^[14]. Bell's staging criteria for NEC are guidelines for the management of NEC (Table 1).

Ideally, nutrition intervention would begin when an infant has one or more risk factors for developing NEC (i.e. preterm birth) or is at an early stage of disease.

Diagnostic methods

Early diagnosis of gut ischemia and mucosal inflammation/necrosis is crucial in the prevention of NEC or the progression of the illness to late stages requiring surgery and/or bowel resection. An abdominal radiograph and a chest x-ray are used to diagnose gastrointestinal tract abnormalities and changes in the size and shape of the lung and heart, respectively^[10,11]. The experimental and clinical methods for early detection of gut ischemia or NEC include serum hexosaminidase, plasma amylin, serum cytosolic β -glucosidase activity, plasma pro- and anti-inflammatory cytokines, serum creatinine kinase isoenzymes, cerebro-splanchnic oxygenation ratio, GI tonometry, rectosigmoid pH monitoring, urinary EGF, D-lactate, or thromboxane, breath hydrogen, and MRI^[8]. Most of these methods do not have high clinical utility either due to accessibility issues, high costs, and need for expert assistance or due to their poor properties as a diagnostic/screening test especially in the early stages of NEC. Some infants present so acutely and severely that morbidity or mortality cannot be avoided despite best treatment. Identification of a biological marker for early disease should allow more timely diagnosis and treatment, but no ideal marker has yet been identified. The serum of symptomatic infants tends to contain high concentrations of certain cytokines such as interleukin-8 (IL-8)^[15]. Some studies suggest that serum concentrations of fatty acid binding protein in the intestine and liver (I-FABP and L-FABP) could also be used as markers for NEC^[16,17]. L-FABP concentrations at the onset of clinical signs are highest in infants later diagnosed with stage I NEC and I-FABP concentrations are highest in infants who later develop stage III NEC^[16,17]. More sensitive and accurate imaging studies, such as ultrasonography, could become helpful adjuncts to abdominal films in the diagnosis of NEC^[18]. Further research is needed on new approaches for the medical management of NEC that might prevent disease progression and improved surgical outcomes to reduce complications such as short bowel syndrome.

PATHOGENESIS

Although the exact etiology and pathogenesis of NEC remains elusive, it is well established that NEC is a

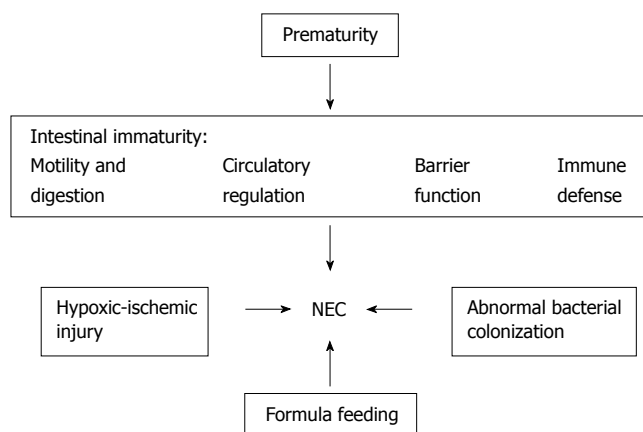


Figure 1 Pathophysiology of necrotizing enterocolitis (NEC).

complex, multi-factorial disease^[2]. Besides pre-maturity, research suggests that other potential predisposing factors are hypoxic-ischemic injury, feeding with formula milk and colonization by pathological bacteria^[1] (Figure 1).

Recent studies have shown that carrier state of genetic polymorphisms may be associated with perinatal morbidity, including NEC^[19]. The hallmarks of NEC are loss of gastrointestinal motility, disruption of intestinal mucosal integrity, and mucosal inflammation, all of which result in the final common pathway, intestinal apoptosis and necrosis^[4,20-23]. Several inflammatory and vasoactive mediators including platelet activating factor (PAF), cytokines, nitric oxide (NO), endothelin-1 (ET-1), prostaglandins, leukotrienes, and reactive oxygen species (ROS) are considered to play a synergistic and central role in the final inflammatory pathway leading to NEC^[20]. The consequent breakdown of the mucosal barrier and impaired ability of the mucosa to heal leads to the self-perpetuating vicious cycle resulting in severe NEC, shock, sepsis, and sometimes death^[8,24,25].

Prematurity

Prematurity is the only factor consistently found in epidemiological studies to be an independent determinant of NEC^[2]. Up to 90% of infants with NEC are of low birth weight and the disease is more frequent and severe in those infants with the earliest post-conceptual age^[7]. The increased susceptibility is attributed to an immature mucosal barrier and barrier response, changing intestinal microflora and increasing enteral volumes^[2,23].

Immature intestinal motility, digestion, and barrier function

Intestinal motility is a critical factor in clearing antigens presented to the intestinal mucosal barrier from the gut lumen. The time available for absorption depends on the speed of luminal contents. Migratory motor complexes act as “house keepers” to propel luminal components caudally along the length of the small intestine. Immature intestinal motility and digestion may predispose preterm infants to NEC. Fetal studies in both animals and humans suggest that development of gastrointestinal motility begins in the second trimester, but matures in the third trimester^[26-28]. Studies of intestinal motility have shown

that premature infants can have immature motility patterns when compared with full-term infants and that maternal-fetal disease states that induce fetal hypoxia can further reduce postnatal intestinal motility^[29-31]. Immature motility patterns alter normal peristaltic activity and result in overgrowth of anaerobic bacteria in the small intestine with malabsorption of dietary nutrients^[23]. In addition, to impaired intestinal motility, premature infants have not yet developed the ability to digest and absorb nutrients and incompletely digested molecules could contribute to intestinal injury^[32,33]. Lebenthal and Lee^[34] showed that the function of the exocrine pancreas is limited in infants and that pancreatic insufficiency may last through the first year of life. Lack of stimulation of gastric acid and pancreaticobiliary secretions and their resulting proteolysis may adversely affect the intestine by allowing a greater bacterial and/or antigenic load. Thus, impaired digestion of nutrients, coupled with delayed transit time and bacterial overgrowth could result in intestinal injury with immature host and barrier defenses.

If structural or biochemical components of the intestinal epithelial barrier are not fully developed, bacteria may gain access to deeper tissues and cause inflammation. Intestinal epithelia are joined by tight junctions that regulate intestinal permeability and form by 10 wk gestation^[35]. Studies show that intestinal permeability to macromolecules including immunoglobulins, proteins, and carbohydrates is highest in premature infants, particularly in those diagnosed with NEC^[20,23]. When fully developed, the intestinal epithelial barrier can allow selective permeability to small ions, absorption of nutrients and control of bi-directional fluid flow. Enterocytes use chloride ions and water secretion to flush unwanted pathogens or toxins from the intestinal lumen. Fetal intestinal secretion and absorption are underdeveloped in preterm infants and mature gradually, under the influence of amniotic fluid, from 26 wk gestation to full-term^[32]. Therefore, pathogens or toxins might not be efficiently washed from the intestinal lumen and could translocate across the leaky intestinal barrier in preterm infants.

Goblet cells are found throughout the small and large intestine. These specialized enterocytes secrete mucins, forming a thick protective layer over the intestinal mucosa. This mucus layer impedes direct microbial-epithelial binding and enhances removal of adherent bacteria^[36]. Preterm infants have immature goblet cells. Developmental expression of mucin genes changes throughout the intestine and matches adult pattern expression between 23 and 27 wk gestation^[37]. Microvilli of immature intestine also have altered glycosylation patterns^[38]. Since carbohydrate sequences are recognition and attachment sites for microbes, changes in glycosylation patterns may influence the bacterial colonization pattern of the gut. An immature mucin layer might lead to increased intestinal permeability and enhanced bacterial adherence, potentially breaching the intestinal epithelial barrier and increasing susceptibility to injury.

Another aspect of the intestinal epithelial barrier that may not be functioning correctly in preterm infants is biochemical defenses. Paneth cells, which are specialized

secretory enterocytes located at the base of small intestinal crypts, secrete lysozyme, phospholipase A₂, and antimicrobial peptides (also secreted by absorptive enterocytes) that regulate composition and distribution of different bacterial populations^[39,40]. Defensins (α and β) and cathelicidins are the two main families of antimicrobial peptides produced by intestinal cells^[40]. These antimicrobial peptides have bioactivity against a wide range of microbes including bacteria, viruses, and fungi^[41]. Some have a pro-inflammatory role and chloride secretory activity^[42,43]. A better understanding of how biochemical defense molecules modulate host immune defenses *in vivo* should contribute to understanding the pathophysiology of NEC.

It is well established that growth factors, growth factor receptors, or their related signal transduction pathways are aberrant in the immature intestine. Epidermal growth factor (EGF) is a major trophic factor for the development of the intestine and the EGF receptor has been identified on the basolateral surface of enterocytes^[44]. Exogenous infusion of EGF *in utero* has been shown to accelerate the maturation of intestinal enzyme activity as well as stimulate intestinal growth^[45]. In the amniotic fluid, there is an increasing concentration of EGF as gestation progresses^[46]. In fact, the salivary level of EGF is directly proportional to the gestational age of the infant^[46]. Moreover, expression of EGF receptor involved in intestinal maturation and restitution is decreased in the preterm infant^[7]. Recently, human data suggests a link between EGF production and NEC. Serum and salivary levels of EGF are significantly reduced in infants with surgical NEC^[47]. Preliminary studies on the clinical use of EGF report improved epithelial regeneration with no significant toxicities^[48].

It is unclear whether the intestinal epithelium of the infant can respond to injury to the same extent as the adult. In animals, infant intestinal epithelium turnover is much slower (4–5 d) than the adult (2 d)^[49]. If the same finding holds true in humans, regeneration of injured mucosa in the infant will be much slower than the adult. Trefoil factor peptides (TFF1–3) are part of the protective mechanism operating in the intestinal mucosa and play a fundamental role in epithelial protection, repair, and restitution^[50]. These secreted peptides have been identified in a site-specific pattern in the gastrointestinal mucosa and their expression has been shown to be up-regulated in early stages of mucosal repair^[51,52]. The role of trefoil peptides in neonatal mucosal protection has not been well investigated. Intestinal trefoil factor is developmentally regulated and deficient in the premature neonate^[20]. Recent studies demonstrated a lack of trefoil factor expression in response to NEC in the premature gut^[53] and an insufficient proliferative response to reverse the mucosal insult observed in NEC^[54]. Thus, impaired restitution of the mucosa may contribute to the cascade of bowel necrosis and generalized sepsis characteristic of NEC.

Immature intestinal immunity

Although the fetus at term may be sensitized to certain antigens, the fetus does lack a fully functional immune

system and has a sterile gastrointestinal tract. Changes occur at, and soon after birth, in order that the immune system of the neonate becomes competent and functional and that the gut becomes colonized with bacteria. Exposure to bacteria during birth and from the mother's skin and the provision of immunological factors in breast milk are amongst the key events that promote maturation of the infant's gut and gut-associated immune system^[55]. Dendritic cells play an important role in the initiation of the immune response. Microbial and antigenic-priming of dendritic cells develops different signals that drive the differentiation of naïve Th cells into Th1, Th2 or T regulatory cells^[56]. Developmental changes in glycosylation patterns of immature dendritic cells may play an important role in development, maturation, and immune regulation^[57].

Innate and adaptive immune defense systems are abnormal in developing neonates^[20]. A possible mechanism for the pathophysiology of NEC is that reduced inflammatory signaling could allow bacterial overgrowth. Newborns are Th2 polarized and do not respond efficiently to IFN- γ ^[58]. Moreover, newborn macrophages exposed to LPS are defective in producing pro-inflammatory cytokines including tumor necrosis factor- α (TNF- α), interleukin-1 β (IL-1 β), interleukin-6 (IL-6) and interleukin-12 (IL-12)^[58,59]. Interestingly, inhibitory activity to toll receptors in neonatal but not adult plasma has been detected^[60]. Neonatal monocyte and T cell production of the anti-inflammatory cytokines interleukin-10 (IL-10) and TGF- β are developmentally delayed^[61]. Preterm infant polymorphonuclear (PMN) counts are lower and premature neonatal macrophages have reduced respiratory burst activation as compared with term newborns^[62]. Under conditions of stress, PMNs of term and preterm infants do not function with normal phagocytic and microbicidal activities^[63]. PMNs isolated from the blood of term and preterm neonates consistently display diminished chemotactic and adhesion capacities^[64]. It is known that intestinal lymphocytes are decreased in neonates (B and T cells) and do not approach adult levels until 3–4 wk of life^[20]. Newborns also have markedly reduced synthesis of secretory IgA and IgG in response to mitogens, reflecting decreased activity in the intestine^[20]. Failure to activate inflammatory pathways in premature infants might prevent induction of anti-apoptotic, cytoprotective factors. Thus, developmental immaturity of the inflammatory response could increase susceptibility to apoptosis when cells are challenged by environmental stress.

Long-term survival requires inflammation as a defense mechanism, however, uncontrolled inflammation results in intestinal barrier damage, translocation of pathogens, further inflammation, and tissue damage. Some *in vitro* studies suggest that immature intestinal cells have a propensity for exaggerated inflammatory responses to pathogenic stimuli and researchers postulate that developmentally deficient expression of the NF- κ B inhibitor I κ B might allow greater NF- κ B activity^[65,66]. NF- κ B is a nuclear transcription factor that enhances the production of inflammatory mediators and is essential for innate immunity, adaptive immunity and cell survival^[67]. In the human newborn, PAF-AH activity is decreased and

Table 2 Ischemic events associated with necrotizing enterocolitis

Perinatal asphyxia
Polycythaemia
Cyanotic congenital heart disease
Patent ductus arteriosus
Medications that ↓ superior mesenteric blood flow (cocaine)
Maternal pre-eclampsia

PAF synthesis pathways are increased. This imbalance places the newborn at special risk of an increased PAF response before adequate immune stimuli are developed^[7].

Hypoxia-ischemia

Pathological findings of NEC associated with ischemic events (coagulative necrosis, Table 2) and the fact that NEC most commonly occurs in the distal ileum and proximal colon, which make up the watershed area of the superior and inferior mesenteric arteries, suggests that derangement of the circulatory system is involved^[7].

Preterm neonates are more susceptible to hypoxia and intestinal ischemia because their system for regulating vascular resistance is poorly developed^[68]. The most distinguishing feature of the newborn intestinal circulation is its very low vascular resistance due to substantial generation of endothelial derived NO when compared with ET-1^[69]. Immature intestine handles the increased metabolic demands of growth by increasing blood flow and oxygen consumption^[10]. However, during episodes of cardiovascular stress, infants are less able to raise intestinal blood flow and metabolic demands overwhelm the infant's ability to increase oxygen consumption^[10]. Defective pressure flow autoregulation in response to hypotension occurs^[68]. Consequently, hypoxia in tissues can occur. Hypoxia increases production of vasoconstrictor ET-1 and ischemia/reperfusion compromises production of endothelial derived vasodilator NO^[69]. Thus, an imbalance between ET-1 and NO production by the newborn intestine following an initial ischemic insult might exacerbate existing intestinal ischemia. Whether the hypoxic/ischemic insult is primary, secondary or both an initiating factor and end result remains controversial. One plausible mechanism that is often cited is the "diving reflex", whereby blood flow is preferentially diverted to the heart and brain in preference to less vital organs^[1]. Very early descriptions regarding the pathogenesis of NEC suggested a primary or early role for ischemia and hypothesized that a hypoxic/ischemic insult directly damaged the intestinal mucosa disrupting the neonatal gut barrier and promoting bacteria translocation and the inflammatory cascade^[21]. Animal models suggest that NEC may not occur without significant reperfusion injury resulting from the generation of oxygen-free radicals at the restoration of blood flow and oxygen delivery after ischemia^[9]. Inflammatory mediators may also cause intestinal ischemia by up-regulating ET-1 production and the expression of its receptor ET_A^[69]. Current studies show a stronger association with prematurity, rapid feeding, abnormal intestinal colonization and inflammatory

mediators than with ischemia^[23]. Hypoxia-ischemia might contribute to the pathogenesis of NEC, but it likely plays more of a secondary role.

Formula feeding

Enteral feeds have a firm association with NEC as 90%-95% of NEC cases occur in infants with initiation/re-initiation of enteral feeds or recent volume advancement^[2,20]. Infants receiving hyperosmolar formulas or rapid volume advancements are at greatest risk^[20]. Although the mechanism is not well understood, enteral feeding has been reported to contribute to the development of NEC through disruption of mucosal integrity, blood flow and motility and through provision of a bacterial substrate^[2,10]. Raising milk intake increases metabolic demands, making it difficult for the infant to expand mesenteric blood flow to meet demands^[10]. As a result, intestinal hypoxemia may occur. Increased proliferation of potentially pathogenic bacteria may go on to invade the bowel wall^[10]. Although the newborn gastrointestinal tract is sterile at birth, bacterial colonization occurs within hours^[10]. Contact with the mother's vaginal flora begins this process, which is further developed by oral feedings and exposure to the environment^[10]. In fact, breast fed infants are 10X less likely to develop NEC than formula fed infants, suggesting that breast milk contains multiple bioactive factors that influence host immunity, inflammation and mucosal protection. Breast milk notably increases the diversity of gastrointestinal bacterial colonization and contains immunomodulatory factors such as secretory immunoglobulin A, leukocytes, mucin, lysozyme, cytokines, lactoferrin, growth factors, enzymes, oligosaccharides, and polyunsaturated fatty acids not provided in commercially available neonatal formula preparations^[20,55]. These factors are capable of inducing mucosal protection and neutralizing potent pro-inflammatory cytokines and phospholipids^[55]. Glutamine and nucleotides may help in gastrointestinal cell metabolism^[10]. EGF can directly improve gastrointestinal function and promote gut maturity^[25].

Abnormal bacterial colonization and infection

The well-documented epidemics of NEC and the improvement in incidence and severity following the implementation of strict infection control measures validates the role of infection in the pathogenesis of NEC^[2]. Furthermore, the regions of the intestine that are most often associated with NEC (ileum and proximal colon) have very high bacterial loads. Moreover, no cases of NEC have been described *in utero*, supporting the importance of bacteria colonization in the pathophysiology of NEC^[20].

Although several bacterial and viral species have been associated with outbreaks of NEC (*Clostridium* sp, *Klebsiella* sp, *Staphylococcus epidermis*, *Escherichia coli*, *Rotavirus*), no single pathogen has been identified as causative and the ability of the microflora to colonize the epithelium and to ferment unabsorbed nutrients may be more important than the strain itself^[13,70]. Recently, early abnormal colonization of stools with *Clostridium perfringens* has been correlated with later development of NEC^[71]. *Clostridium perfringens* has

been isolated from 40% of infants with NEC, compared with 13% of controls^[71]. Premature infants are especially susceptible to intestinal colonization by pathological bacteria due to their daily exposure to nosocomial flora and the likelihood of exposure to antibiotics and steroids on admission to NICUs^[72].

Colonization of the gastrointestinal tract of the premature infant differs greatly from that of the healthy term infant^[9,20]. Patterns of intestinal colonization also vary according to the type of enteral feeding^[3]. The colonization of the hospitalized, premature infant gastrointestinal tract has less species diversity and fewer anaerobic species of *Lactobacillus* and *Bifidobacterium*^[9,20]. Breast-fed infants have large amounts of protective, gram-positive *Bifidobacteria* in their intestine, contrasting with formula-fed neonates who are colonized predominantly by potentially pathogenic gram-negative *Enterobacteria*^[3]. Gram-positive bacteria yield lactic acid during carbohydrate metabolism, which is readily absorbed from the intestinal lumen, whereas gram negative-bacteria ferment lactose into hydrogen, carbon dioxide and organic acids, producing distension, increased intraluminal pressure, decreased mucosal blood flow and pneumatosis intestinalis^[3]. Enteral feeds and poor gastrointestinal motility associated with immaturity may promote stasis and bacterial overgrowth^[3]. This microbial imbalance may represent a fertile environment for the pathologic overgrowth, binding and invasiveness of otherwise non-pathogenic intestinal bacterial species capable of triggering the inflammatory cascade with resultant NEC^[9]. Recently, inappropriate immunologic responses of premature enterocytes to bacteria colonization have been implicated in the development of NEC^[13]. Reports indicate that pathogenic stimuli including *Salmonella* and *E. coli*, produce exaggerated pro-inflammatory responses in immature intestinal epithelial cells^[65,66].

Abnormal expression of pattern recognition receptors that recognize microbial signatures might also affect the way in which the intestine in premature infants responds to bacterial colonization. One of the first pro-inflammatory molecules to cross the intestinal barrier is lipopolysaccharide (LPS), which is a principal component of the outer cell wall of Gram-negative bacteria that recognizes and binds to toll like receptor 4 (TLR4)^[21]. Circulating LPS is increased in patients with NEC, which inhibits epithelial restitution and initiates inflammatory signaling cascades within the enterocyte including activation of transcription factor NF- κ B and expression of enzymes that produce apoptotic NO and pro-inflammatory eicosanoids and cytokines^[21] (Figure 2).

In rats, intestinal epithelial cells up-regulate expression of TLR4 in response to stress-induced production of PAF, suggesting that up-regulation of TLR4 might explain how NEC develops in this animal model^[73]. It remains unclear whether bacterial translocation into submucosa is a prerequisite for disease or whether the activation of the Toll-like receptors from endotoxin and other bacterial cell wall products is adequate to initiate the final common pathway of intestinal injury^[20]. For premature infants at risk for NEC, there may be increased passage of bacteria

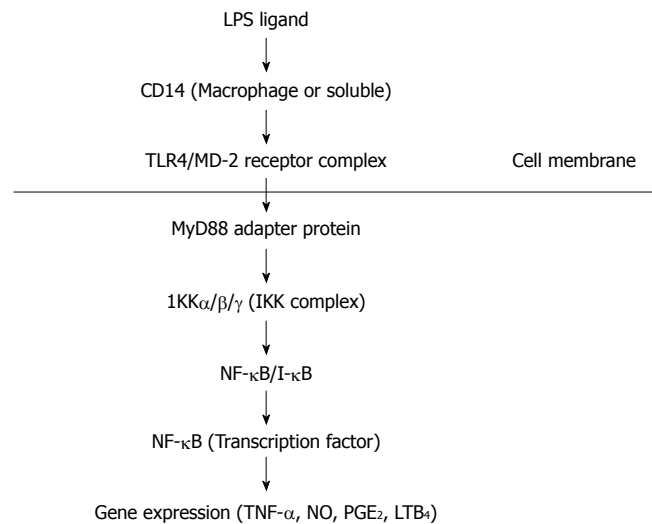


Figure 2 LPS-Induced signaling pathways leading to NF- κ B activation.

from the gut into the systemic circulation and exaggerated pro-inflammatory responses^[10]. Most of the defenses that would normally prevent passage of bacteria across the mucosal barrier—a well-functioning immune system, intact mechanical defenses and normal intestinal microflora are impaired in patients who are at risk for NEC^[10]. Gram-negative bacteria translocate to regional lymph nodes and activate resident macrophages to release inflammatory mediators^[2]. Bacteria endotoxins can leak into the systemic circulation causing release of inflammatory mediators, intestinal damage, shock and death^[2,10].

Commensal bacteria interact symbiotically with the mammalian intestine to regulate the expression of genes important for barrier function, digestion, and angiogenesis^[74]. Commensal bacteria can inhibit inflammatory pathways and perhaps contribute to the maintenance of homeostasis^[75]. *In vitro* experiments show that a wide range of commensal bacteria can reduce inflammatory signaling in intestinal epithelia by inhibition of the NF- κ B signaling pathway^[76,77]. Preliminary work suggests that early colonization by probiotics (facultative anaerobes such as *Lactobacilli* and *Bifidobacteria*) reduces the risk of NEC in very low birth weight infants^[78,79].

Genetics

Investigation of factors that might cause a genetic predisposition for NEC might eventually allow specific treatments or preventative strategies for the infants most at risk for this disease. Current technology allows for the detection and evaluation of genetic polymorphisms and their influence on disease development. Studies are now emerging which investigate the potential importance of specific polymorphisms for known NEC-associated inflammatory mediators. The presence of genetic variance may contribute to the inter-individual variance of cytokine response to inflammatory stimuli^[19]. A family of intracytoplasmic pathogen recognition receptors has been shown to sense invading bacteria and activate gene transcription pathways that regulate immune and inflammatory responses. In a recent clinical study, VLBW

infants with mutations in a member of this family, NOD2, demonstrated increased susceptibility to bacterial sepsis^[80]. Genetic polymorphisms of CD14, TLR4, and NOD2 are not associated with NEC in VLBW infants^[81]. In preliminary studies, VLBWI with NEC were shown to be less likely to possess the interleukin-4 (IL-4) receptor α -chain mutant allele compared to infants without NEC^[19]. The investigated variant of IL-4 receptor α gene is associated with enhanced transduction of IL-4 signals which shifts the development of lymphocytes to a more pronounced Th2 state^[19]. It is speculated that the elevated number of Th2 cells in carriers of this genetic polymorphism is a protective factor against the development of NEC^[19]. The risk of NEC has also associated with the frequency of the IL-18⁶⁰⁷ AA genotype. The frequency of the AA genotype is significantly higher in infants with stage 3 NEC compared to stages 1 and 2^[19]. Thus, the presence of the AA genotype may adversely affect the outcome of NEC through altered IL-18 levels, a cytokine that induces IFN- γ and amplifies Th1 cytokine production and IL-8 accumulation^[19]. Another possible genetic factor is the pro-inflammatory cytokine TNF- α . In animal models, pretreatment with anti-TNF- α reduces the incidence and severity of NEC^[82,83]. Investigators have not reported a genetic link between TNF- α gene variants and the disease^[84].

Vasoactive and inflammatory mediators

Bacterial colonization and enteral feeds coupled with damage to and loss of the integrity of the immature gastrointestinal mucosa trigger the final common pathway leading to the development of NEC^[9]. Inflammatory mediators are responsible for protecting the body from invading organisms and play a vital role in the pathogenesis of NEC^[3]. Inflammation can be initiated by a variety of factors including exposure to the bacterial cell wall product, endotoxin, and ischemia reperfusion^[20]. The release of potent biologically active phospholipids, cytokines, products of arachidonic acid metabolism, vasoactive mediators, neurotransmitters, and reactive oxygen species from the immature and damaged gastrointestinal cells and inflammatory cells amplify the inflammatory response, leading to tissue damage and NEC^[9]. Studies of animals and human cell lines suggest that the balance between pro-inflammatory and anti-inflammatory modulatory factors in premature infants is pro-inflammatory^[9].

NO

NO is a short-lived, labile free radical gas that reacts with a variety of biologically active substances^[85]. Such reactions result in both local and systemic effects that modulate the inflammatory response in a variety of tissues^[21]. The synthesis of NO in biological systems is regulated by nitric oxide synthase (NOS), which catalyzes the oxidation of the amino acid L-arginine to release citrulline and nitric oxide^[21]. Although diverse molecular reactions of NO have been identified in physiological and pathological systems, the fastest and most biologically relevant reaction of NO is with superoxide to produce the potent oxidant peroxynitrite^[21]. Peroxynitrite is a

key intermediate that is generated at inflammatory sites and is responsible for mediating tissue injury, in part, through lipid peroxidation^[21]. Three isoforms of NOS exist: Neuronal (nNOS) and endothelial (eNOS), which are calcium/calmodulin dependent and constitutively expressed, releasing physiologically low concentrations of nitric oxide (pM) and the calcium independent inducible isoform (iNOS), which releases toxic concentrations of nitric oxide (nM) in response to infection and inflammatory stimuli^[86]. All three isoforms are expressed in the gastrointestinal tract^[21,86]. The constitutive forms are expressed by endothelial cells, enteric neurons, gastric epithelial cells, and enterocytes^[86]. In the gastrointestinal tract, NO mediates inhibitory nerve-related relaxation of intestinal smooth muscle and plays a role in regulating gut mucosal blood flow, mucosal permeability, intestinal motility and mucosal protection^[85,86]. Normal smooth muscle sphincter function as well as coordinated peristalsis is dependent on the integrity of intrinsic nitric oxide neurons of the myenteric and submucosal networks throughout all regions of the gut wall^[85]. NO also maintains intestinal microvascular integrity by inhibiting platelet aggregation and leukocyte adhesion^[86]. Ontogenic variation in constitutive NOS activity has been observed in different animal species, in humans and in different organs^[85]. By contrast, iNOS expression and activity within the intestinal epithelium is normally low, although it may be increased 15-fold after 4 h stimulation with LPS^[21]. NO and peroxynitrite have anti-microbial properties and play important roles in host defense against pathogens^[86]. However, sustained high levels of NO production promote bacteria translocation following insults such as endotoxemia and ischemia-reperfusion injury^[86]. The induction of iNOS mRNA expression by inflammatory mediators has been seen in animal models of NEC and in intestinal resections from patients with NEC where the predominant source of iNOS activity was the enterocytes. Endothelial NOS function is compromised in human intestine resected for NEC^[87]. Poorly coordinated production of NO by NOS isoforms occur during the early phase of the disease and are involved in altered intestinal blood flow, ischemic damage, disassembly of tight junction proteins, and impaired healing typically seen in NEC^[13]. Research suggests that NO participates in the pathogenesis of NEC by directly damaging the enterocyte monolayer and by disrupting the ability of the mucosa to repair itself^[21]. Extensive apoptosis has been shown in the enterocytes of the apical villi of infants with NEC and this correlates with the degree of nitrotyrosine immunostaining, a marker of NO release and tissue reactivity^[88]. Toxic concentrations of NO have also been shown to decrease enterocyte proliferation and inhibit enterocyte migration^[21]. It is proposed that peroxynitrite interferes with EGF receptor signaling in enterocytes^[89].

ET-1

ET-1, a potent vasoconstrictor agent, is produced at several sites within the intestine including vascular endothelial cells, submucosal stroma, and circularis muscularis layers of the gut wall^[69]. Although constitutively produced,

ET-1 production is increased by a wide range of stimuli including reduced flow rate, hypoxia and inflammatory cytokines^[69]. ET-1 generates a profound degree of ischemia that is sustained for hours because of a unique interaction between ET-1 and its receptor^[69]. If not balanced by concomitant vasodilatory stimuli, ET-1-induced ischemia can generate hypoxia and tissue death^[69]. ET-1 induces vasoconstriction by binding to ET_A receptors present within the newborn intestine and whose activation can generate intestinal tissue damage when excessive amounts of ET-1 are present^[69]. Recently, ET-1 was demonstrated to be associated with NEC. It has recently been shown that the tissue concentration of ET-1 is greater in human preterm intestine that demonstrates histologic evidence of NEC^[90]. Moreover, it has been demonstrated that arterioles harvested from intestine exhibiting histologic evidence of NEC exhibits vasoconstriction and that the vasoconstriction can be reversed by blocking ET_A receptors^[90].

Serotonin

Serotonin is an intermediate product of tryptophan metabolism and is primarily synthesized and released by enterochromaffin cells of the intestine (90%) and enteric/brain neurons (10%) in response to calcium influx, physical mucosal stimulation, nutrients, hypoxia, and elevations in intraluminal pressure^[91]. Levels of serotonin in the gastrointestinal tract are regulated by a serotonin uptake transporter, SERT, present in the mucosa and enteric nerves^[92]. The major function of serotonin in the gastrointestinal tract is stimulation of bowel motility, epithelial secretion, and vasoconstriction through serotonin receptor binding^[91]. Disruption of serotonin homeostasis and signaling is commonly seen in several gastrointestinal motility and inflammatory disorders including bowel obstruction and inflammatory bowel disease^[93]. In inflammatory bowel disease, serotonin levels and enterochromaffin cell numbers are increased^[93]. The inflamed intestinal tissue releases more serotonin, has a reduced capacity to remove serotonin and the serotonin receptors are desensitized^[93]. Some cases of NEC have been associated with maternal use of paroxetine, a long-acting serotonin re-uptake inhibitor^[94].

PAF

PAF, an endogenous phospholipid with powerful pro-inflammatory actions, is synthesized by neutrophils, macrophages, endothelial cells, and enterocytes in response to endotoxin and hypoxia^[10]. PAF formation begins with the conversion of a phosphatidylcholine precursor to a biologically inactive intermediate, lysoPAF, under the influence of cytosolic phospholipase A₂^[73]. Subsequent acetylation of lysoPAF at the n-2 position by acetyltransferase completes PAF synthesis^[73]. PAF has a very short half life as it is rapidly degraded by PAF-acetylhydrolase^[73]. In the human newborn, PAF synthesis pathways are increased and the activity of the PAF-degrading enzyme PAF-acetylhydrolase is decreased^[7]. This imbalance places the newborn at special risk of an elevated PAF response before adequate immune stimuli are

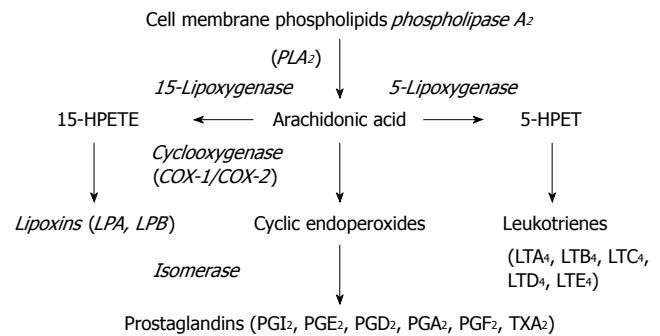


Figure 3 Metabolic pathways of arachidonic acid and eicosanoid production.

developed^[7]. Formula does not contain PAF-AH like human milk, leaving susceptible neonates at greater risk for NEC. PAF exerts its effects by binding to PAF receptors present on most cells^[73]. Interestingly, PAF receptors are most highly concentrated in the ileum, the region of the intestine where NEC is very prominent^[24]. Down-stream signaling includes elevation of cytoplasmic free calcium and stimulation of protein kinase C, mitogen-activated protein kinase (MAPK), and NF- κ B with production of inflammatory molecules including iNOS, TNF- α , ET-1, IL-1, IL-6, and IL-8^[73]. PAF also activates pathways that result in caspase activation and apoptosis^[73]. PAF is one of the most extensively studied mediators of intestinal injury and has been indicated as an important mediator in several animal models and human analyses of NEC^[4]. PAF infusion causes intestinal necrosis in animals and PAF receptor antagonists prevent injury following hypoxia, endotoxin challenge and ischemia reperfusion injury^[20]. Human patients with NEC show high levels of PAF and decreased levels of plasma PAF-AH with levels correlating with NEC severity^[4,24]. In immature or mildly damaged mucosa, the close proximity of bacteria and intestinal epithelial cells aids transcellular permeation of PAF into the mucosa and local entry of bacteria^[10]. Injection of LPS and bacterial invasion leads to increased production of platelet activating factor, release of secondary inflammatory mediators and further mesenteric ischemia and damage causing clinical NEC^[10,24].

Eicosanoids

Arachidonic acid is a polyunsaturated fatty acid that is liberated from cell membrane phospholipids and serves as a precursor for many immune active lipids, collectively called eicosanoids (oxygenated C20 fatty acids)^[95,96]. Classes of eicosanoids that signal in the immune system include prostaglandins, leukotrienes and lipoxins^[95]. The major producers of eicosanoids are platelets, monocytes, macrophages, neutrophils, and mast cells, although with the exception of leukotrienes, they are also synthesized by a variety of non-immune cell types^[95]. These lipid mediators are not stored in cells rather they are synthesized from arachidonic acid via three major metabolic pathways, either constitutively or in response to cell-specific trauma, stimuli, or signaling molecules^[96] (Figure 3).

The 15-lipoxygenase metabolic pathway results in the production of 15-hydroxyperoxy-eicosatetraenoic acid (15-HPETE) that serves as a precursor for the lipoxins

Table 3 Eicosanoid synthesis and actions

Eicosanoid	Cell/tissue origin	Target cell/tissue	Receptor	Action
PGE2	Most cells	Many cells	EP1-EP4	Fever, pain
PGI2	Endothelium	Platelet VSMC	IP	Declumping, vasodilation
PGF2	Uterus	Uterine SMC	FP	Contraction
PGD2	Mast cells	Lung Th2 cells	DP1/DP2	Asthma, chemotaxis
TXA2	Platelets	Platelet VSMC	TP α /TP β	Aggregation, vasoconstriction
LTB4	Macrophage monocytes	Neutrophils	BLT1/BLT2	Promotes chemotaxis
LTC4/LTD4/LTE4	Macrophage monocytes	Lung SMC	BLT3/BLT4	Bronchoconstriction
LXA4	Leukocytes	Neutrophil	LXA4 R	Inhibits chemotaxis
LXB4	Leukocytes	NK cells	?	Inhibits cytotoxicity

LPA and LPB. Lipoxins exert anti-inflammatory activities through stimulation of macrophage phagocytosis of apoptotic neutrophils and inhibition of natural killer (NK) cell cytotoxicity and pro-inflammatory factor production^[97,98].

Prostaglandins are end products of metabolism of arachidonic acid by constitutive and inducible cyclooxygenase isoforms (COX-1 and COX-2, respectively)^[96]. The COX-1 enzyme accounts for basal prostaglandin synthesis for homeostatic regulation while COX-2 is involved in the synthesis of pro-inflammatory prostaglandins^[96]. Leukotrienes are generated during the metabolism of arachidonic acid by the 5-lipoxygenase pathway and exert pro-inflammatory effects^[95]. Prostaglandins and leukotrienes are emitted from their cell of origin and exert their effects in an autocrine or paracrine fashion by signaling through specific G-protein coupled receptors^[95] (Table 3).

Cytokines

Pro-inflammatory cytokines are multifunctional proteins produced in response to inflammatory stimuli that communicate to the surrounding tissue the presence of infection or injury. Several pro-inflammatory cytokines that mediate inflammatory cell recruitment through activation and amplification of the immune response in local host defense have been implicated in NEC including TNF- α , IL-1 β , IL-6, IL-8, IL-12, and IL-18^[99-101]. Anti-inflammatory cytokines modulate the host's inflammatory response and if they fail to achieve their goal, pro-inflammatory mediators can continue, resulting in tissue injury^[4]. The anti-inflammatory cytokines IL-4 and IL-10 have been implicated in NEC^[4].

Granulocyte-Macrophage Colony Stimulating Factor (GM-CSF): The colony stimulating factors are a group of cytokines central to the hematopoiesis of blood cells, the modulation of their functional responses as well as the maintenance of homeostasis and overall immune competence^[102]. GM-CSF is produced by a variety of cell types including macrophages, T lymphocytes, fibroblasts, endothelial cells, B lymphocytes, mast cells, eosinophils, and neutrophils^[103]. In some cases, production of GM-CSF is constitutive as in a number of tumor cells lines; however, in most cases it requires stimulation of the producing cell with other cytokines, antigens, or inflammatory agents^[103]. All of the biological effects of GM-CSF are mediated via the GM-CSF receptor which signals through

MAPK and JAK/STAT pathways^[103]. GM-CSF receptor expression is characterized by low number (20-200 per cell) and high affinity^[103]. GM-CSF has pleiotropic and widespread effects on hematopoietic cells. It functions to promote the proliferation and maturation of neutrophils, eosinophils, and macrophages from bone marrow progenitors^[103]. It also acts as a growth factor for erythroid and megakaryocyte progenitors in synergy with other cytokines^[103]. The role of GM-CSF in cell survival results from apoptosis inhibitory mechanisms^[103]. In addition to its role in the up-regulation of hematopoietic development, GM-CSF has been shown to have a profound influence on the biological functions of neutrophils, eosinophils, basophils, macrophages, lymphocytes, as well as endothelial cells^[103]. These responses are widespread and point to a central role of GM-CSF in inflammation both through the direct activation of effector cells alone or in combination with other cytokines, as well as indirectly, through the stimulation of additional inflammatory mediator production^[103]. Some of these biological effects include enhanced antigen presentation, chemotaxis, synthesis of a variety of soluble mediators and enzymes, release of reactive oxygen intermediates and histamines, antibody-dependent cell killing, and phagocytosis which contribute differentially to the immune defenses against bacterial, viral, fungal, and parasitic infections as well as tumor development^[103]. Over-expression of GM-CSF leads to severe inflammation^[104]. GM-CSF is used clinically to treat neutropenia in cancer patients undergoing chemotherapy, in AIDS patients during therapy and in patients after bone marrow transplant^[103,105].

TNF- α : TNF- α release is triggered by a number of inflammatory stimuli including endotoxin (LPS), gram positive bacteria enterotoxin, viruses, fungi, and parasites^[106]. Important cell sources of TNF- α in the gut are macrophages, lymphocytes, NK cells, neutrophils, endothelial cells, smooth muscle cells, intestinal epithelial cells, and enteric glia^[100,106]. TNF- α exerts its effects by binding to TNF receptors^[107]. Binding to the TNF receptor initiates local inflammatory responses through cell activation^[107]. TNF- α is released early following injury and leads to a cytokine release cascade of IL-1 β , IL-6, and IL-8^[100]. It also inhibits release of glucocorticoids and the regulatory cytokines TGF- β and IL-10^[106]. Some actions mediated by TNF- α include apoptosis induction, neutrophil activation, neutrophil recruitment, expression

of endothelium adhesion molecules, fever, and production and release of acute phase proteins, pro-inflammatory cytokines, NO, PGE₂, matrix metalloproteases, PAF, and TXA₂^[106]. The pro-inflammatory effects of TGF- α are mediated in part through NF- κ B activation^[106]. Elevated TNF- α has been detected in full thickness, resected bowel specimens of NEC intestine and in the plasma of babies with NEC^[101]. In rat models of NEC, TNF- α induces hypotension, septic shock and severe intestinal necrosis synergistically with LPS^[24]. Recently, a monoclonal anti-TNF- α antibody was demonstrated to reduce hepatic and ileal TNF- α production in a neonatal rat model of NEC^[82]. Compared with other inflammatory bowel syndromes, TNF- α transcripts are lower in NEC^[108]. Furthermore, studies indicate that the majority of TNF- α found in the gut lumen comes from Kupffer cells in the liver^[99]. Taken together, these studies suggest that TNF- α plays a less significant role in the inflammatory cascade associated with NEC as compared with other intestinal inflammatory conditions.

IL-1 β : IL-1 β release is triggered by a variety of stimuli including microbial products, inflammation and TNF- α ^[100,109]. Important cell sources of IL-1 β in the gut are macrophages, neutrophils, intestinal epithelial cells, endothelial cells, fibroblasts, dendritic cells, smooth muscle cells, and enteric glia^[100,109]. IL-1 β exerts its effects by binding to the IL-1 receptor and activating the transcription factor NF- κ B^[109]. Some actions mediated by IL-1 β include macrophage activation, neutrophil recruitment, expression of endothelium adhesion molecules, fever, and production and release of acute phase proteins, IL-6, IL-8, and PGE₂^[100,109]. Elevated IL-1 β has been detected in full thickness specimens of NEC intestine^[101]. Studies measuring plasma/serum IL-1 β in NEC babies have not consistently reported elevated levels^[15]. The difference in results may suggest that IL-1 β is more predominant in the intestinal tissue in patients with NEC.

IL-6: IL-6 release is triggered by a variety of stimuli including microbes, microbial products, TNF- α , and IL-1 β ^[100,110]. Important cell sources of IL-6 in the gut are macrophages, endothelial cells, and intestinal epithelial cells^[110]. IL-6 exerts its effects by binding to the IL-6 receptor that signals through the STAT-4 pathway^[110]. The IL-6 receptor is only expressed on hepatocytes and some leukocytes^[110]. Some actions mediated by IL-6 include production of acute phase proteins, B cell growth, antibody production, T cell proliferation, and enhanced activity of hematopoietic growth factors such as GM-CSF^[100,110]. Anti-inflammatory effects of IL-6 include production of tissue inhibitors of metalloproteinases and inhibition of superoxide production^[111]. High levels of umbilical cord IL-6 have been associated with neonatal disease processes including NEC and systemic inflammatory response syndrome^[112]. Elevated IL-6 has been reported in the plasma and stool of babies with NEC^[4]. A study that looked at IL-6 mRNA expression in surgical intestine specimens from babies with NEC did not

find a difference in comparison to control specimens^[101]. Since IL-6 plays a dual role in inflammation it may serve as an anti-inflammatory mediator despite being correlated with increased morbidity and mortality in NEC patients.

IL-8: IL-8 synthesis and release is triggered in response to various stimuli including LPS, TNF- α , and IL-1 β ^[113]. Important cell sources of IL-8 in the gut are macrophages, endothelial cells, intestinal epithelial cells, and fibroblasts^[114]. IL-8 exerts its effects by binding to chemokine receptors CXCR1 and CXCR2 that signal through phospholipase c and PI3-kinase, respectively^[113]. Some actions mediated by IL-8 are attraction of neutrophils and basophils to the site of inflammation, neutrophil activation and migration into tissues and production of acute phase proteins^[114]. In intestinal specimens from patients with NEC, IL-8 mRNA expression was up-regulated, correlated with disease severity and was limited to areas with histological inflammation^[4,115]. Similarly, plasma IL-8 levels are elevated in infants with NEC and levels correlate with clinical severity^[4]. The vulnerability of the premature infant to develop NEC may, in part, be explained by the excessive inflammatory response shown by fetal enterocytes compared to more mature enterocytes^[66]. When exposed to inflammatory stimuli, fetal intestinal cells exhibit a greater IL-8 response compared to mature intestinal cells^[66]. This exaggerated response may partly be explained by the developmental down regulation of I- κ B, an inhibitor of NF- κ B^[65].

IL-12: IL-12 synthesis and release is the early response to bacteria, bacterial products, and viruses^[116]. Important cell sources of IL-12 in the gut are macrophages, neutrophils, B cells, and dendritic cells^[116]. IL-12 exerts its effects by binding to IL-12 receptors present on T cells and NK cells^[116]. Some actions mediated by IL-12 include IFN- γ production, Th1 and NK cell proliferation, cytotoxic T lymphocyte and Th1 cell differentiation, macrophage activation, and production of complement-fixing antibodies^[116]. Several studies have identified putative NF- κ B sites in the promoter regions of the IL-12 p40 genes^[117]. IL-12 is a potentially important cytokine in the development of NEC. Halpern^[99] localized IL-12 via immunohistochemistry to monocytes in the intestinal mucosa and lamina propria and correlated IL-12 positive cells with tissue damage in a neonatal rat model of NEC.

Interleukin-18 (IL-18): IL-18 is a cytokine that shares structural and functional properties with IL-1 and is pro-inflammatory inducing production of TNF- α and IL-1 β ^[118]. IL-18 synthesis is triggered by LPS, Fas ligand and gram positive bacteria exotoxins^[119]. Important cell sources of IL-18 in the gut are macrophages, dendritic cells, and intestinal epithelial cells^[119]. IL-18 exerts its effect by binding to the IL-18 receptor present on macrophages, neutrophils, NK cells, endothelial cells, smooth muscle cells, and lymphocytes^[119]. IL-12 upregulates the IL-18 receptor on lymphocytes^[119]. Binding to the IL-18 receptor results in NF- κ B activation. Some actions mediated by IL-18 include IFN- γ production,

enhanced NK cell cytotoxic activity, B cell antibody production, macrophage production of IL-8, activation and migration of neutrophils, phagocytosis, and integrin expression^[119]. IL-18 can promote Th1 or Th2 lineage maturation depending on the underlying genetic influence and cytokine environment. The risk of NEC has been associated with the frequency of the IL-18⁶⁰⁷ AA genotype^[19]. Recent data imply that IL-18, in the absence of IL-12, may facilitate the development of Th2 responses^[118]. IL-18 is also essential to host defense against a variety of infections^[119] and is potentially important in the development of NEC. Immunohistochemistry reveals the presence of IL-18 in intestinal epithelial and lamina propria cells which correlates with the degree of tissue damage in a neonatal rat NEC model^[99]. Depending on the surrounding environment, IL-18 may play a destructive or protective role in NEC.

IL-4: IL-4 is a pleiotropic, immunoregulatory cytokine produced by Th2 cells, mast cells, B cells, and stroma cells^[100,120]. IL-4 displays a wide variety of effects on B cell growth and differentiation, T cell growth and regulation, hematopoietic cells and differentiation of CD4⁺ T cells into Th2 cells and is a key regulator in humoral and adaptive immunity^[100,120]. IL-4 induces B cell class switching to IgE and upregulates MHC class II production^[100,120]. IL-4 is known to promote Th2 type responses and to exert immunosuppressive effects on macrophages including the suppression of pro-inflammatory cytokine production^[100,120]. Although data are not available about the importance of IL-4 in NEC, isolated lamina propria mononuclear cells from the inflamed intestinal mucosa of children with chronic inflammatory bowel disease express and secrete IL-4 in lower amounts than control cells^[121]. In preliminary studies, VLBWI with NEC were shown to be less likely to possess the IL-4 receptor α -chain mutant allele compared to infants without NEC^[19]. The investigated variant of IL-4 receptor α gene is associated with enhanced transduction of IL-4 signals which shifts the development of lymphocytes to a more pronounced Th2 state^[19]. It is speculated that the elevated number of Th2 cells in carriers of this genetic polymorphism is a protective factor against the development of NEC^[19].

IL-10: IL-10 is the most important regulatory cytokine in the intestine and is primarily synthesized by Th2 cells, monocytes, and B cells^[120]. Mononuclear production of anti-inflammatory mediators such as IL-10 is diminished in the newborn when compared to the adult, with preterm infants synthesizing less than term infants^[122,123]. It is postulated that this phenomenon allows for persistent up-regulation of the inflammatory response and therefore increased susceptibility in the preterm neonate to long-term tissue damage after acute inflammatory conditions^[124]. Interleukin-10 has been implicated as an inhibitor of pro-inflammatory cytokine production and of several accessory cell functions of the macrophage, T cell and natural killer (NK) cell lines^[120]. Kuhn^[125] demonstrated that IL-10 deficient knockout mice were predisposed to developing inflammatory colitis, suggesting that IL-10 works to counterbalance the response

to enteric inflammatory stimuli. In fact, intraperitoneal IL-10 injections in a mouse model of ischemia/reperfusion injury reduced local and systemic inflammatory reactions^[126]. Edelson^[15] noticed significantly increased concentrations of IL-10 with severe NEC. IL-10 has also been shown to decrease the production of metalloproteinases^[127] and suppress iNOS mRNA and NO expression in small bowel, liver and serum^[128]. These findings indicate that IL-10 is a strong counter regulatory cytokine and that the potential of IL-10 to provide therapy in the setting of NEC is high. Perhaps the high levels of IL-10 in severe NEC are the body's response to dampen the inflammatory response.

ROS

One of the major endogenous sources of ROS in the intestine is the xanthine dehydrogenase/xanthine oxidase (XD/XO) system^[129]. Xanthine dehydrogenase (XD), the precursor of XO, is constitutively and abundantly expressed in the intestinal villus epithelium, which catalyzes the conversion of hypoxanthine to xanthine, coupled with the reduction of NAD⁺ to NADPH^[130]. Because XO uses molecular oxygen rather than NAD⁺ as an electron acceptor and thereby generates superoxide, XD to XO conversion (during ischemia) has been suggested to play the central role in intestinal reperfusion injury^[129]. Following PAF challenge, it is the ileum that shows the most dramatic XD to XO conversion^[130]. The central role of XO and ROS in causing the injury is supported by pre-treatment with allopurinol, a xanthine oxidase inhibitor, which largely prevents PAF-induced bowel necrosis^[131]. Infusion of superoxide dismutase plus catalase also alleviates the injury^[131]. In a piglet model of NEC, the level of the tissue antioxidant, α -tocopherol (vitamin E) was low in formula compared to colostrum fed piglets^[13]. Thus, infants with NEC are under oxidative stress and may benefit from exogenous sources of antioxidants to replenish limited supplies.

NEC models

There are a number of accepted models used to study NEC and the cytokine cascade. These models serve to create necrotic bowel in animals to simulate that in the newborn child. LPS, PAF and TNF- α are often used to create intestinal ischemia. LPS is thought to mimic the bacterial overgrowth in the intestinal lumen and PAF and TNF- α cause a hypotensive response and shock^[24].

Many animal models can simulate NEC, but often do not contain the aspect of prematurity that is seen in human NEC. The most physiological animal model that most closely resembles human NEC entails removing rat pups from the maternal uterus, exposing them to maternal milk, and stressing them with asphyxia, gram negative bacteria colonization, and artificial formula feedings^[132]. After a few days of life, the rat pups begin to exhibit signs of NEC including intestinal distension and bloody diarrhea.

Other models have been described that do not physiologically resemble human NEC, but aid in the study of the disease process. These include inducing hypoxia for 5 min followed by 10 min with 100% oxygen^[133], hypoxia for 50 s followed by cold exposure^[134], superior

mysenteric artery clamping with or without PAF^[135], intraarterial injection of TNF- α ^[136], and placing rats into a 100% nitrogen or 10% oxygen environment^[24]. Finally, a rat model has been described by Chan^[137] who created intestinal ischemia by increasing intraluminal pressure and injecting *E. coli* into the lumen.

In addition to *in vivo* animal models, various *in vitro* models have been created. The cell lines are often intestinal-derived and immortal such as CaCo-2, a human colon carcinoma cell line^[138]. Inflammatory stimulants such as LPS and pro-inflammatory cytokines can be added to cell cultures which can then be analyzed to determine the presence or absence of specific cytokines. In addition, cells can be studied with regards to permeability, viability and expression of inflammatory markers after addition of certain stimulants or creation of hypoxic environments. Paracellular conductance can be assessed by measuring both trans-epithelial resistance (TER) and determining the rate of permeation of radiolabelled, hydrophilic probes between mucosa and serosa compartments of vertical diffusion chambers. It is unfortunate that primary cultures of human enterocytes have a limited life span (hours) in culture and therefore have not been useful as a model.

Symptomatic treatment and surgery

Due to the limited understanding of the fundamental biological processes that underlie the development of NEC, there is no cure for this devastating pediatric disease^[21]. Symptomatic treatment of the infant with NEC begins with prompt recognition of the diagnosis and medical stabilization^[2,9]. The treatment of NEC is based on the severity of the disease and is directed toward reduction of factors that aggravate the disease, treatment of infection and support of respiratory and cardiovascular systems^[139]. Blood pressure should be closely monitored, all enteral feedings and medications should be discontinued and decompression of the gastrointestinal tract with placement of a gastric tube should proceed to evacuate residual air and fluid^[9]. Rapid volume expansion with isotonic fluids may be necessary to reverse hypotension as well as frequent monitoring of blood glucose levels^[9]. An intravenous infusion of total parenteral nutrition should begin during the 10-14 d bowel rest period^[9]. The reinstitution of feedings generally is done in a slow and cautious manner, using an elemental formulation to allow for optimal absorption of all nutrients and to avoid further potential injury to the intestinal mucosa^[7]. Broad-spectrum antibiotics including ampicillin and an aminoglycoside should be started as soon as cultures have been obtained^[139]. With the increasing prevalence of infections from coagulase-negative staphylococcus, vancomycin may be used instead of ampicillin^[2]. Anti-microbial choices should be guided by local resistance patterns^[2,139]. Adjunctive therapy includes cardiovascular support (volume expansion with packed red blood cells), pulmonary support (oxygen and ventilation), and hematological support (blood product transfusion) as clinically indicated^[1,139]. Indications for surgical intervention include peritoneal free air and signs of intestinal perforation^[9]. Surgical intervention

Table 4 Strategies to prevent necrotizing enterocolitis

Evidence-based support for efficacy	Limited data to support efficacy
Breast feeding	Cautious advancement of feedings
Trophic feeding	Fluid restriction
Antenatal steroids	Oral immunoglobulins
Enteral administration of antibiotics	L-arginine supplementation
	Polyunsaturated fatty acids
	Acidification of milk feeds
	Probiotics, prebiotics and postbiotics
	Growth factors and erythropoietin
	Free radical scavengers

frequently results in resection of areas of necrotic bowel and exteriorization of viable ends (multiple ostomies) to allow for continued bowel decompression^[2,9]. Recently, primary peritoneal drainage has been proposed as an alternative to surgical treatment. NEC STEPS and NET, prospective multi-centre randomized controlled trials, are currently underway to examine the effectiveness of primary peritoneal drainage versus laparotomy as primary therapy for perforated NEC in VLBW infants^[140,141].

PREVENTION

Strategies to prevent NEC fall into two major categories: Those with probable or proven efficacy and those that are experimental with unproven efficacy^[2] (Table 4).

The most effective preventative strategies should improve both short-term and long-term outcomes for VLBW preterm infants and address the problems of prematurity.

Human milk

Human milk has been reported to reduce the incidence of NEC by up to 10 fold compared with infant formula whether using mother's own or donor milk^[142]. Breast milk also reduces the severity of NEC^[8]. The protective effect of breast milk has been correlated with its anti-inflammatory components (IL-10), growth factors (EGF), erythropoietin (Epo), lysozyme, immunoglobulins as well as pre- and probiotics that modulate intestinal microflora composition to the advantage of the host^[55,143,144]. Research looking at a gut-stimulation, or gut-priming protocol has demonstrated potential benefits of promoting maturation of the gut by introducing early feedings with human milk^[3]. The activity of acetyl hydrolase (PAF-AH), an enzyme that degrades PAF, is lower in neonates under 3 wk of age than at any other time^[145,146]. The additional presence of PAF-AH activity may also partly explain the protective effect of breast milk, as infant formulas do not contain it^[8]. Whether preterm human milk reduces the incidence of NEC is not clear at present^[8]. Despite its advantages, it is important to appreciate that human milk alone will not eliminate NEC as cases are reported in neonates who have been breast-fed exclusively with human breast milk^[8].

Trophic feeds

Initiation of trophic feeds, small volumes of breast milk

or formula, may overcome gut atrophy and inflammatory responses associated with prolonged bowel rest. Trophic feeds improve the activity of digestive enzymes, enhance the release of digestive hormones and increase intestinal blood flow and digestive motility in premature infants^[147]. In addition, infants given early trophic feeds seem to have better feeding tolerance, improved growth, reduced period of hospitalization and decreased likelihood of sepsis compared with infants who are not^[147]. Furthermore, early trophic feeds do not increase susceptibility to developing NEC. However, studies have not yet clearly delineated the best feeding strategies for premature infants^[147].

Antenatal glucocorticoids

Antenatal glucocorticoid therapy has beneficial effects by suppressing inflammation and promoting gastrointestinal maturation and function including reduced mucosal uptake of macromolecules, decreased colonization with aerobic bacteria, reduced bacterial translocation, and increased activity of enzymes such as lactase, maltase, sucrase, and Na/K-ATPase^[148,149]. A significant reduction in the incidence and risk of NEC following antenatal glucocorticoid therapy has been reported in several large, randomized control trials^[150,151]. Mortality rate was also lower and there were fewer indications for surgical intervention^[152]. Antenatal glucocorticoids have been reported to alter immune system development in very premature infants^[153]. Mothers with the presence of infection or a condition that may compromise blood flow to the fetus (ex. pre-eclampsia) during pregnancy may be at risk of delivering a premature baby and may potentially benefit from early use of glucocorticoids. Thus, antenatal glucocorticoid therapy is a simple and effective strategy for global prevention of NEC and more research should be done to investigate potential impact on development.

Enteral antibiotics

Enteral antibiotics have been used as prophylaxis against NEC in low birth weight and preterm infants given the role of bacterial colonization in the pathogenesis of the illness. A systemic review and meta-analysis has reported that the administration of prophylactic enteral antibiotics resulted in significant reduction in NEC^[154]. The trend towards a reduction in deaths was not significant^[154]. The possible harmful effects of prophylactic antibiotics including the development of bacterial resistance and alteration of the natural microflora make it difficult to recommend this strategy for prevention of NEC.

Standardized feeding regimens (cautious advancement of feedings)

Inter-centre differences in clinical practice involving feeding regimens are significant factors linked to the prevalence of NEC in VLBW neonates^[155]. A relationship between the rate of feeding advancement and an increased incidence of NEC exists^[156]. A significant decline of 87% in the incidence of NEC and 29% in the risk of developing NEC was reported following implementation of a standardized feeding regimen in the form of clinical practice guidelines^[157,158]. Parenteral nutrition coupled

with minimum enteral feeding is the approach commonly advocated for the initial nutritional management of high risk infants and helps protect against NEC^[159].

Fluid restriction

Excess fluid has been implicated in the pathogenesis of NEC. A systemic review and meta-analysis indicates that restricted water intake significantly increases postnatal weight loss and significantly reduces the risk of NEC^[160]. Careful restriction of water intake (meeting the physiological needs without allowing significant dehydration) could be expected to decrease the risk of death from NEC without significantly increasing the risk of adverse consequences.

Probiotics

Since bacterial colonization can affect the course of many intestinal diseases, probiotics are emerging as a promising therapy. Probiotics are living microorganisms, which upon administration in sufficient numbers colonize the gut and exert health benefits beyond basic nutrition on the host^[161]. As components of infant formula, typically used probiotic microorganisms are members of the genera *Lactobacillus*, *Bifidobacterium*, *Saccharomyces* and to a lesser extent *Streptococcus*. The beneficial effects of probiotics range from changes in intestinal permeability and enhanced mucosal IgA responses to an increased production of anti-inflammatory cytokines and protection of the mucosa against colonization from pathogens^[162]. *Bifidobacteria* are the most common organisms recovered from the gastrointestinal tract of breast-fed neonates. Given the role of inappropriate gastrointestinal colonization by bacteria in the pathogenesis of NEC, probiotics may be beneficial in the prevention of NEC. Several studies have used different strains of probiotics and different administration regimens (length of treatment and dose) in preterm infants. None of the trials have reported adverse effects and no episodes of pathogenic infection caused by a probiotic organism have been observed^[178,79,163,164]. Clinical trials show that probiotic supplements (*Lactobacillus acidophilus*, *Bifidobacterium infantis*, *Bifidobacterium bifidus*, and *Streptococcus thermophilus*) reduce the incidence and severity of NEC^[78,79,165]. Larger clinical trials are necessary to confirm the safety and efficacy of this promising intervention to better define the benefits and risks for premature infants before wider use can be recommended.

Prebiotics

Another potential preventative strategy is to administer prebiotics, non-digestible dietary supplements, such as long chain carbohydrates or mucins, which promote proliferation of beneficial commensal bacteria^[166]. Preliminary studies show increased *Bifidobacterium* stool colonization and decreased pathogenic bacterial colonization in preterm infants fed with formula containing prebiotics (90% short chain galacto-oligosaccharide, 10% long chain fructo-oligosaccharide) compared with infants fed control formula^[167]. Furthermore, prebiotic treatment may have a positive effect on host immune function^[168]. Because prebiotic supplements do not contain live microorganisms, they carry less risk of infection than

probiotic therapies. However, prebiotic administration has been associated with unwanted (but reversible) side effects such as flatulence, bloating and diarrhea^[166].

Postbiotics

Another potential therapy involves bacterial metabolites or postbiotics, such as butyric acid, a short-chain fatty acid produced by commensal bacteria in the colon through anaerobic catabolism of complex carbohydrates. Butyrate is a major energy source for colonic enterocytes and has a widely recognized but poorly understood role in intestinal growth and differentiation^[169,170], inflammatory suppression^[171] and apoptosis^[172]. Butyrate and other small molecule products might generate some of the beneficial effects of the normal flora (and exogenous probiotics and prebiotics), and could be a safe alternative therapeutic strategy. Butyrate has been administered with limited success in human inflammatory bowel disease^[173], but there are as yet no studies in neonates.

Other products of commensal bacteria can induce protective responses that promote intestinal health. The beneficial effects of probiotic bacteria can be replicated by treatment with isolated microbe-associated molecular patterns (MAMPs)^[174]. A MAMP is a molecular sequence or structure in any pathogen-derived molecule that is perceived via direct interaction with a host defense receptor^[175]. For example, in mice unmethylated probiotic DNA ameliorates colitis^[174]. Oral administration of inactivated probiotics (heat-killed commensals) or bioavailable toll-like receptor ligands could potentially induce beneficial TLR-mediated protective effects without carrying the infectious risk of probiotic therapies.

Arginine supplementation

Endothelial nitric oxide is an anti-inflammatory agent and vasodilator that is involved in the maintenance of intestinal vascular permeability, mucosal integrity and barrier function^[21,86]. The plasma levels of the amino acid arginine, a substrate for NOS, have been shown to be low in neonates with NEC^[176,177]. Arginine supplementation has recently been shown to reduce the incidence of all stages of NEC in a randomized, double blind, placebo controlled trial in preterm neonates^[178]. Whether the beneficial effects of arginine supplementation in prevention of NEC are related to synthesis of glutamine or to its free radical scavenging action is currently unknown^[179,180]. Guidelines have not been established for the safety and efficacy of L-arginine at doses above standard dietary practices in NEC^[181].

Free radical scavengers (anti-oxidants)

Free radicals have been implicated in several neonatal disease processes including NEC^[182]. A human recombinant superoxide dismutase is currently available and has been shown to prevent damage and attenuate eicosanoid release in a rabbit model of NEC^[183,184]. The anti-oxidant vitamin E has been shown to reduce lipid peroxidation and intestinal lesions in a neonatal rat model of NEC induced by hypoxia-ischemia^[183,184]. More studies on the therapeutic role of anti-oxidants in NEC should be done.

Acidification of gastric contents

Preterm neonates are often hypochlorhydria and enteric, Gram negative bacteria often colonize their stomachs, especially after gavage feeding^[185]. Carrion and Egan^[186] have documented that acidifying the feedings of preterm neonates to a pH low enough to inhibit gastric bacterial proliferation significantly lowers the risk and incidence of NEC.

Polyunsaturated fatty acids

Phosphatidylcholine (PC) is a major phospholipid constituent of mucosal membranes and the fatty acid component of PC, arachidonic acid, is a substrate for intestinal vasodilatory and cytoprotective eicosanoids^[8]. Long chain polyunsaturated fatty acids (PUFA) have been proposed to modulate inflammation and immunity^[187]. A clinical trial of formula feeds with or without supplementation with PUFA in the form of egg phospholipids in preterm neonates showed that the supplemented formula contained 7-fold more arachidonic acid and docosahexanoic acid and reduced the incidence of stage II and III NEC^[188]. Recent evidence from an experimental study indicates that the protective effect of long chain PUFA is mediated by modulation of PAF metabolism and endotoxin translocation^[189].

Oral immunoglobulins

A number of reports have been published, which suggest that oral immunoglobulins (IgA and IgG) have an immunoprotective effect on the gastrointestinal mucosa^[190,191]. Premature infants have decreased levels of immunoglobulins, especially secretory IgA^[192]. A reduction in the incidence of NEC following feeding an oral IgA-IgG preparation was reported as early as 1988^[190]. A systemic review on oral immunoglobulin for the prevention of NEC did not show a significant reduction on the incidence of definite NEC^[193]. No randomized controlled trials of oral immunoglobulins for the prevention of NEC have been carried out. Current evidence does not support the administration of oral immunoglobulin for the prevention of NEC.

EGF

EGF is a growth factor that exerts its effects by binding to the EGF receptor. EGF is an important constituent of gastrointestinal secretions and has multiple effects upon gut epithelial cells including cytoprotection, stimulatory effects on cell proliferation and migration, induction of mucosal enzyme and trefoil peptide expression, and inhibitory effects on gastric acid secretion^[48]. Preterm neonates with NEC have diminished levels of salivary and serum EGF^[194]. The presence of immunoreactive EGF receptors in gut epithelial cells of preterm neonates with NEC raises the possibility of using EGF for prophylaxis or treatment of NEC^[195]. In a neonatal rat model of NEC, EGF treatment maintained intestinal integrity at the site of injury by accelerating goblet cell maturation and mucin production and normalizing expression of tight junction proteins^[196]. Researchers have already warranted that the clinical use of EGF may be associated with a variety of problems and side

effects and that careful selection of patients and evaluation of risk-benefit ratios are necessary^[197]. Given the potential for adverse effects and the fact that maturity alone is not a protective factor for NEC the use of any growth factors in preterm neonates warrants extreme caution.

Epo

The presence of Epo in human milk and the expression of Epo receptors on intestinal villous enterocytes of neonates suggest that Epo has a role in growth and development of the gastrointestinal tract^[198-200]. Ledbetter^[200] administered recombinant Epo for the prevention and treatment of the anemia of prematurity and demonstrated that the rEpo group had a lower incidence of NEC. Akisu^[133,201] indicated that rEpo decreased lipid peroxidation but not PAF generation. Although not completely absorbed, Epo acts as a trophic factor in developing rat bowel whether given enterally or parenterally^[199]. Current evidence indicates that the protective effect of rEpo may be related to inhibition of NO formation^[202].

CONCLUSION

A variety of other experimental agents have been studied in search for an effective agent for the prevention of NEC. These include anti-TNF- α ^[82], PAF receptor antagonists^[203], heparin-binding EGF-like growth factor^[204], anti-inflammatory cytokines (IL-10)^[205], pentoxifylline^[206], intestinal trefoil factor 3^[207], and glucagon-like peptide 2. Recent research has identified that complex glycosphingolipids in the form of gangliosides act as bioactive factors down-regulating many acute pro-inflammatory signals in the intestinal mucosa. Perhaps the solution to NEC will involve identification of an intestinal control mechanism that optimizes (or disregulates) the balance between pathways that signal inflammation, hypoxia, and mucosal cell growth or metabolism.

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GASTRIC CANCER

Effect of *NHE1* antisense gene transfection on the biological behavior of *SGC-7901* human gastric carcinoma cells

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potential role for human tumor gene therapy.

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Abstract

AIM: To study the effect of type 1 Na⁺/H⁺ exchanger (*NHE1*) antisense human gene transfection on the biological behavior of gastric carcinoma cell line *SGC-7901*.

METHODS: Antisense *NHE1* eukaryotic expression on vector pcDNA3.1 was constructed by recombinant DNA technique and transfected into gastric carcinoma cell line *SGC-7901* with DOTAP liposome transfection method. Morphological changes of cells were observed with optic and electron microscopes. Changes in cell proliferative capacity, apoptosis, intracellular pH (pH_i), cell cycle, clone formation in two-layer soft agar, and tumorigenicity in nude mice were examined.

RESULTS: Antisense eukaryotic expressing vectors were successfully constructed and transfected into *SGC-7901*. The transfectant obtained named *7901*-antisense (*7901-AS*) stably produced antisense *NHE1*. There was a significant difference between the pH_i of *7901-AS* cells (6.77 ± 0.05) and that of *7901-zeo* cells and *SGC-7901* cells (7.24 ± 0.03 and 7.26 ± 0.03, *P* < 0.01). Compared with *SGC-7901* and *7901-zeo* cells, *7901-AS* cells mostly showed cell proliferation inhibition, G₁/G₀ phase arrest, increased cell apoptotic rate, recovery of contact inhibition, and density contact. The tumorigenicity in nude mice and cloning efficiency in the two-layer soft agar were clearly inhibited.

CONCLUSION: *NHE1* antisense gene significantly restrains the malignant behavior of human gastric carcinoma cells, suppresses cell growth and induces cell apoptosis, and partially reverses the malignant phenotypes of *SGC-7901*. These results suggest a

INTRODUCTION

The type 1 Na⁺/H⁺ exchanger (*NHE1*) is a transmembrane protein found in all eukaryotic cells. One of its functions is to remove excess H⁺ in the cytoplasm by Na⁺-H⁺ exchange system, resulting in a stable intracellular pH level^[1,2]. The process of glycolysis induces the tumor cells to produce large quantities of lactic acid and H⁺. There is vigorous Na⁺-H⁺ exchange in the tumor cells, dependent upon the enhanced expression of *NHE1* membrane protein. Most of the H⁺ pumped out of the cells prevents the intracellular acidification of tumor cells, resulting in the protection of tumor cells from apoptosis^[3,4]. Previous studies have shown that *NHE1* protein expression in gastric carcinoma tissues was significantly higher compared to that in normal gastric mucosa and precancerous lesions^[5]. Therefore, inhibition of up-regulation of *NHE-1* gene expression in human gastric carcinoma cells may induce intracellular acidification, resulting in apoptosis of tumor cells, which is helpful in the treatment of such tumors. In the present study, we constructed the antisense *NHE-1* eukaryotic expression vector and transfected it into gastric carcinoma cell line *SGC-7901* in order to investigate the effects of antisense *NHE1* on malignant biological behavior of the gastric carcinoma cell line *SGC-7901*.

MATERIALS AND METHODS

Cell line and cell culture

The human gastric carcinoma cell line *SGC-7901* was

used in this study. The cells were grown in RPMI1640 medium (Sigma, St Louis, MO, USA), supplemented with 10% FBS (fetal bovine serum) and antibiotics (100 µg/mL streptomycin and 100 U/mL penicillin) in an atmosphere consisting of 5% CO₂ in air at 37°C in an humidified incubator.

Gene transfection

pEAP cloning vector of human *NHE1* cDNA was kindly provided by Dr. Josset Noel (Montreal University, Canada). Eukaryotic expression vector *pcDNA3.1* (-)/*Zeo* and Zeocin were obtained from Invitrogen Co. The experimental procedures of gene transfection were carried out according to the directions of DOTAPTM liposome transfection kit (Roche Diagnostics, Mannheim, Germany). The cells were plated at a density of $1.5-3 \times 10^5$ cells/35 mm dish and were grown for 24 h. The cells were transfected for 6 h with 2.5 µg plasmid DNA and 15 µL DOTAP in 2 mL of RPMI1640 medium without FBS and antibiotics. The cells were recovered for 48 h in the medium with 10% FBS. The stable transfectant was maintained in 100 µg/mL Zeocin (Invitrogen) in the medium for at least 20 d. The antisense *NHE1* and the control plasmid transfectant were named *7901-AS* and *7901- zeo* , respectively.

Analysis of exogenous genes integration

In order to identify exogenous integration in the nucleic DNA of *SGC-7901* cells, the Zeomycin-resistant antibiotic-selecting gene (*ZeoR*) was amplified by polymerase chain reaction (PCR) assays with a *ZeoR*-specific primer set (5'-GGCCAAGTTGACCAGTGC-3' as forward and 5'-GTCAGTCCTGCTCCTCGG-3' as backward). DNA was extracted from *SGC-7901* and the transfected cells using standard techniques. The total volume of the PCR reaction system was 25 µL, containing 0.2 mmol/L dNTP, 1 mmol/L of each primer, 2U *Taq* polymerase, 1 × reaction buffer and 100 ng DNA template. After predegeneration at 94°C for 5 min in a PE cycle, 30 PCR cycles were performed at 94°C for 30 s, 53°C for 30 s, and 72°C for 30 s, then extended at 72°C for 5 min in a PCR thermocycler (Pekin-Elmer Cetus, Norwalk, CA, USA). Electrophoresis was performed on 1% agarose gel, and the findings were observed and pictures taken under the Burdick lamp.

Determination of intracellular pH

High-concentration potassium-buffer containing the following chemicals (in mmol/L): 90-130 KCl, 5 NaCl, 1 MgSO₄, 1 CaCl₂, and 10 HEPES was infused into 6 tubes (5 mL in each), with pH values adjusted to 5.5, 6.0, 6.5, 7.0, 7.5, and 8.0, respectively. Nigericin (30 µmol/L) and BCECF (0.25 µmol/L) were added to the 6 tubes. At the logarithm growth phase, the cells were digested with pancreatin to prepare a single-cell suspension. The cells were washed twice with PBS, and an identical number of cells were added to the 6 tubes for inoculation at 37°C for 12 min. The cells were stimulated with argon ion laser at 488 nm. The emitted fluorescence was recorded at 530 nm and 640 nm and the ratio (FIR) was calculated. The ratio curve and the standard curve of pH were drawn.

Cells at the logarithm growth phase were digested with

pancreatin, and centrifugated for 5 min at 1000 r/min. After removal of the supernatant, the cells were washed once using saline. BCECF/AM stock solution (2 mg/mL), prepared with anhydrous DMSO, was added to the serum-free and phenolsulfonphthalein-free medium until the BCECF concentration reached 0.25 µmol/L. Cells in the stock solution were incubated for 12 min for 1 h at room temperature. The cells were stimulated with argon ion laser at 488 nm and the emitted fluorescence at 530 and 640 was recorded, and the ratio (FIR) was calculated. Intracellular pH (pH_i) was calculated according to the standard curve.

Cell morphology and growth features observation

The shape, size and growth features (such as contact inhibition, density inhibition and anchorage-dependence) of *7901-AS* cells were observed using invert microscopy and common microscopy after hematoxylin and eosin staining. Cell proliferation speed was assayed by the 3-(4,5-dimethyl-2 thiazoyl)-2,5-diphenyl-2H-tetrazolium bromide method.

Cell cycle and apoptosis rate analysis

Exponentially growing cells were collected and fixed with 75% cold ethanol for at least 24 h and were analyzed for cell cycle distribution and apoptosis rate by DNA content analysis using propidium staining under flow cytometry (FACS420).

Double-deck soft agar colony forming efficiency

The cells were planted in 24-hole plastic plates spread with low melting point agar (0.35% in upper layer and 0.6% in low layer). Each type of cell was planted in five holes, with 1000 cells in each hole. The cells were cultivated at 37°C, with 5% CO₂ and under saturation humidity for 2-3 wk. Cells that were larger than 75 µm in diameter or clones with more than 50 cells were counted under an invert microscope. Clone form-rate was calculated according to the clone form number/inoculation cell number × 100%.

Tumorigenicity assay in nude mice

Nine Balb/c athymic 4-5 wk old female nude mice, bred in specific pathogen free conditions (purchased from the Experimental Animal Center of the Third Military Medical University, Chongqing, China) were randomly divided into three groups. Three different cell lines (*SGC-7901*, *7901- zeo* , *7901-AS*), suspended in 0.1 mL serum-free RMPI 1640, were injected subcutaneously in a dose of $4-8 \times 10^6$ cells at two different sites of the mice. The tumor diameter was measured every 7 d. The animals were monitored regularly for tumor occurrence and size for at least 2 mo.

Statistical analysis

The findings are depicted as mean ± SD. Variance analysis and *t*-test for non-matched data were performed using a professional SPSS statistical program.

RESULTS

Construction and identification of *NHE1* antisense eukaryotic expression vector

Human *NHE1* cDNA (3.6 Kb) digested from plasmid

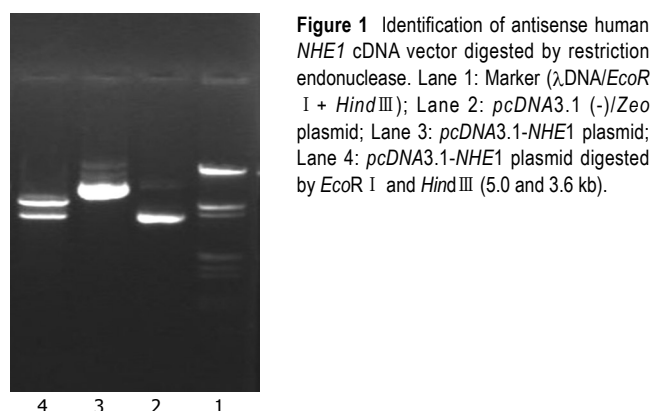


Figure 1 Identification of antisense human *NHE1* cDNA vector digested by restriction endonuclease. Lane 1: Marker (λ DNA/*EcoRI* + *HindIII*); Lane 2: *pcDNA3.1* (-)/Zeo plasmid; Lane 3: *pcDNA3.1-NHE1* plasmid; Lane 4: *pcDNA3.1-NHE1* plasmid digested by *EcoRI* and *HindIII* (5.0 and 3.6 kb).

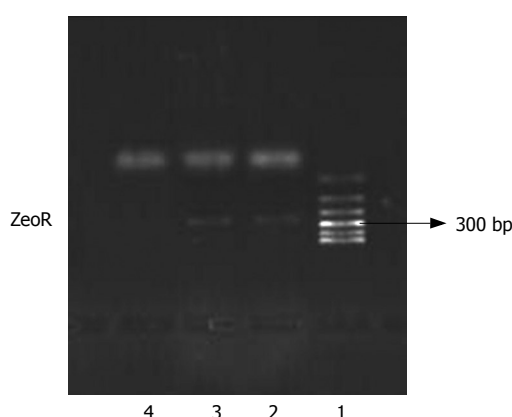


Figure 2 Analysis of exogenous gene integration in nucleic DNA of SGC-7901. Lane 1: Polymerase chain reaction marker; Lane 2: 7901-zeo; Lane 3: 7901-AS; Lane 4: SGC-7901.

pEAP with *EcoRI* and *HindIII* restriction endonuclease was inserted into the eukaryotic expression vector in antisense orientation. The recombinant DNA was further shown to be the same as designed by restriction analysis (Figure 1). A fragment of 3.6 Kb was released after digestion of the recombinant plasmid with *EcoRI* and *HindIII*, suggesting that the target fragment was successfully inserted into the expression vector.

The antisense *NHE1* eukaryotic expression vector was named *pcDNA3.1-NHE1*.

Identification of transfection

We introduced an antisense *NHE1* cDNA sequence into the SGC-7901 cell line. Following Zeocin selection, drug-resistant individual clones were randomly collected from cultures infected with *pcDNA3.1-NHE1* (7901-AS). For controls, drug-resistant clones were selected from the cultures infected with an empty vector *pcDNA3.1* (-)/Zeo (7901-zeo). The expression of zeocin resistant gene (*ZeoR*) could be amplified in 7901-AS or 7901-zeo cells, but not in untransfected cells. This result suggested that exogenous genes had been integrated into the nucleic DNA of SGC-7901 cells (Figure 2).

Determination of intracellular pH

The standard curve drawn according to the buffer is shown in Figure 3. The regression equation was $y = 0.2144x - 0.4248$ ($r = 0.96$).

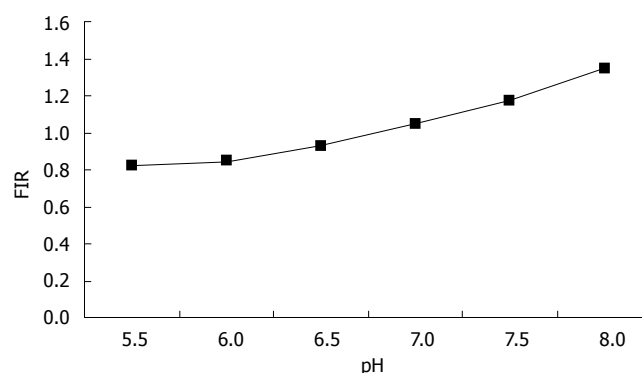


Figure 3 Standard curve of intracellular pH.

Table 1 Changes in the intracellular pH values of SGC-7901 gastric carcinoma cells before and after transfection

Cells	Intracellular pH values determined each time			Intracellular pH (mean \pm SD)
	1	2	3	
7901-AS	6.73	6.75	6.83	6.77 \pm 0.05 ^b
7901-zeo	7.20	7.26	7.25	7.24 \pm 0.03
SGC-7901	7.22	7.28	7.28	7.26 \pm 0.03

^b $P < 0.01$ vs 7901-zeo and SGC-7901.

The cells were stimulated with argon ion laser at 488 nm and the emitted fluorescence at 530 nm and 640 nm was recorded and the ratio of 530/640 was calculated. The intracellular pH (pH_i) was calculated according to the standard curve. The pH_i of 7901-AS cells was 6.77 ± 0.05 , whereas the pH_i of 7901-zeo and SGC-7901 cells were 7.24 ± 0.03 and 7.26 ± 0.03 , respectively. There was a significant difference between the pH_i of 7901-AS cells and that of 7901-zeo cells and SGC-7901 cells ($P < 0.01$). The reduced pH_i of 7901-AS cells suggested that the transfected antisense *NHE1* gene successfully inhibited the expression of *NHE1* gene and blocked excessive exchange of Na^+ - H^+ , resulting in intracellular acidification. There was no significant difference in the pH_i of SGC-7901 and 7901-zeo cells (Table 1).

Morphology and growth features of 7901-AS cells

Compared with their parental cells and 7901-zeo cells, antisense *NHE1*-transfected 7901 cells displayed several morphological changes under light microscopy, such as decreased mitotic figures, multinucleate giant cells, giant nucleate cell numbers, and nucleus:cytoplasm ratio. The 7901-AS cells only grew in a monolayer and at low cell density. The cell proliferation slowed down on the sixth day when 7901-AS cells grew into complete confluence (Figures 4 and 5). Further study showed that the parent cells and 7901-zeo cells grew in clusters with large numbers of big clones. The formation rate was 11% for SGC-7901 cells and 9.5% for 7901-zeo cells after 2 wk culture in soft agar. By contrast, the 7901-AS cells were scattered in soft agar with less number of clones and the formation rate was only 2%.

Cell-cycle distribution and apoptotic rate of 7901-AS cells

The 7901-AS cells showed increased apoptotic cells and

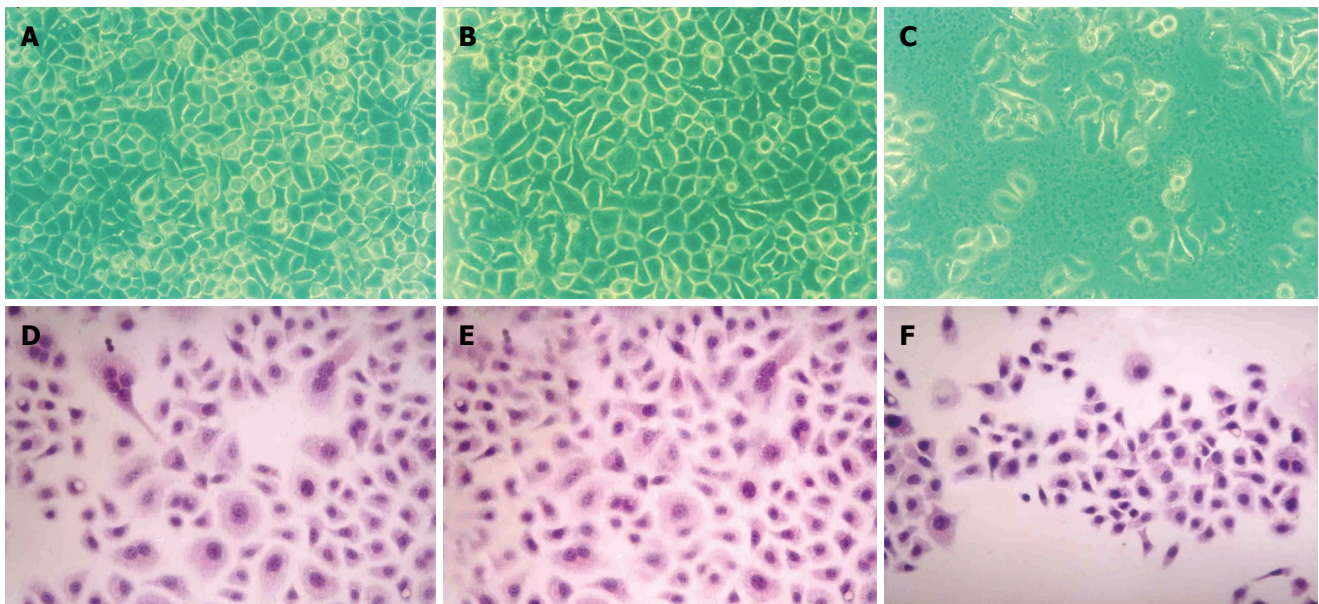


Figure 4 Morphological changes in the transfected cells and parental cells. The first row was observed under inverted microscope. The second row was stained with hematoxylin and eosin, and examined by light microscopy. (A) and (D) SGC-7901; (B) and (E) 7901-zeo; (C) and (F) 7901-AS.

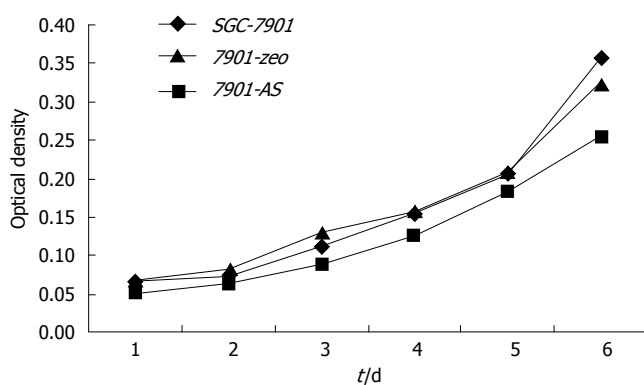


Figure 5 Comparison of growth curves of transfected cells and parental cells.

G₀/G₁ cells, and decreased S and G₂M cells, and reduced proliferative index compared to SGC-7901 and 7901-zeo cells, suggesting that transfection of SGC-7901 cells with antisense *NHE1* gene resulted in leftward shift of the cell cycles and reduced capacity for differentiation and proliferation. Flow cytometry showed apoptotic peak of 7901-AS cells with apoptotic rate of 26.1%, which was significantly higher than that of SGC-7901 cells (4.5%) and 7901-zeo cells (5.1%), suggesting that transfection with antisense *NHE1* induced apoptosis of SGC-7901 cells (Table 2).

Tumorigenicity assay in nude mice

After inoculation with 4×10^6 SGC-7901 cells or 7901-zeo cells, all mice ($n = 3$) grew palpable tumors on the sixteenth-seventeenth day. Subsequently, the tumors grew progressively. The size and speed of growth of the two different tumors showed no apparent difference at 2 mo. After inoculation with 4×10^6 and 8×10^6 dose of 7901-AS cells, none of the mice ($n = 3$, respectively) grew palpable tumors within 2 mo. This result indicates that

Table 2 Cell cycle distribution and the apoptotic rate in transfected and untransfected cells

Cell type	Cell cycle distribution (%)				Apoptotic rate
	G ₀ /G ₁	S	G ₂ /M	PI	
7901-AS	60.0	34.3	6.3	40.3	26.1 ^b
7901-zeo	54.6	39.0	7.6	46.6	5.1
SGC-7901	55.4	37.9	8.0	45.2	4.5

^bP < 0.01 vs SGC-7901 and 7901-zeo.

antisense *NHE1*-transfected SGC-7901 cells completely lose tumorigenicity in nude mice.

DISCUSSION

Since the successful cloning of human *NHE1*cDNA in 1989 by the Sardet Laboratory^[6], the 8 known subtypes of NHE, named as NHE1-8 respectively, have been shown to be identical in structure and are related in function. These form the gene family of membrane exchange protein^[7-10]. The different subtypes have different number and distribution, different pharmacological properties, and are regulated by different factors. *NHE1*, a house-keeping gene, located at 1p^[1] with mRNA of 3.8 Kb, is found in nearly all human tissues, and serves to remove excessive H⁺ in the cytoplasm by the Na⁺-H⁺ exchange system, resulting in the maintenance of a stable pH_i^[11-14]. Glycolysis of tumor cells may result in the production of large quantities of lactic acid and H⁺. Researchers have long believed that the pH_i of tumor cells is more acidic than that of normal cells. However, ³¹P-NMR spectroscopic studies of tumor cells have shown that the pH_i measured *in situ* is more alkaline (pH 7.0-7.2) than that of the normal cells (pH 6.5-7.0). This phenomenon of high pH_i with low extracellular pH (pH_e) is caused by the activation of NHE1 in the cell membrane with increased mRNA expression,

resulting in strong $\text{Na}^+\text{-H}^+$ exchange in the tumor cells. Most of the H^+ pumped out of the cells helps to create an acidic environment in the interstitial fluid of the tumor cells and maintains the pH_i in the tumor cells as neutral or more basic^[15-18]. Our previous studies have shown that the significantly greater expression level of NHE1 protein in gastric carcinoma tissues compared to that in normal gastric tissues is closely associated with the genesis and progression of tumors^[5], suggesting that *NHE1* can be used as the target site in the treatment of such tumors. However, further studies are needed to determine whether or not the intervention of antisense *NHE1* gene can decrease type 1 Na^+/H^+ exchanger of membranous ion exchange protein in gastric carcinoma cells and reverse the malignant phenotypes. Therefore, in the present study, the purpose of transfection of antisense *NHE1* gene into human gastric carcinoma cell line *SGC-7901* was to investigate the effects of antisense therapy targeting *NHE1* gene in the malignant phenotypes of gastric carcinoma cells. It was observed that reduced pH_i partially reversed the malignant phenotypes of *SGC-7901* cells transfected with antisense *NHE1*. Compared with *SGC-7901* and *7901- α eo* cells, the *7901-AS* cells showed cell proliferation inhibition, G_1/G_0 phase arrest, increased cell apoptotic rate, recovery of contact inhibition and density contact, decreased invasive capacity, and loss of cloning efficiency in soft agar, and tumorigenicity in nude mice. These results indicate that the *NHE1* gene is important in maintaining the phenotypes of the *SGC-7901* cell line. The *NHE1* gene may be closely associated with the malignant biological behavior of the tumor cells, and as a result, the phenotype of the tumor is restrained when the *NHE1* gene is inhibited.

Overexpression of *NHE1* gene plays an important role in the regulation of pH_i in tumor cells^[19-26]. The enhancement of $\text{Na}^+\text{-H}^+$ exchange by tumor cells, caused by increasing the quantitative distribution of NHE1 on the cell membrane, is the major molecular mechanism in the regulation of pH_i in tumor cells. This step is of important biological significance in the maintenance of stable pH_i and malignant growth of the tumor cells. The energy consumption process of $\text{Na}^+\text{-H}^+$ exchange which is dependent on the energy supplied by $\text{Na}^+\text{-K}^+\text{-ATPase}$, stimulates glucose absorption and glycolysis by tumor cells and produces more intracellular H^+ , leading to strong $\text{Na}^+\text{-H}^+$ exchange in the tumor cells^[27-30]. Most of the H^+ pumped out of the cells helps to create the acidic environment in the interstitial fluid of the tumor cells and keeps the pH_i neutral or more alkaline in tumor cells, resulting in the protection of the tumor cells from apoptosis^[18,23]. In the present study, transfection of antisense *NHE1* gene inhibited the expression of *NHE1* gene in *SGC-7901* gastric carcinoma cells, resulting in intracellular acidification and induced apoptosis of *SGC-7901* cells. The inhibited proliferation, increased apoptotic rate, and decreased malignancy in tumor cells resulted in significantly reduced tumorigenicity in nude mice *in vivo*. These results indicate that transfected human *NHE1* antisense gene successfully inhibited $\text{Na}^+\text{-H}^+$ exchange and destroyed the energy metabolism pattern of gastric carcinoma cells. Our findings point to the feasibility

of treatment of gastric carcinoma by induction of cell apoptosis through the process of intracellular acidification, which may provide a new method for gene therapy of gastric carcinoma.

In conclusion, we transfected *NHE1* antisense into *SGC-7901* cells and observed the morphological and biological changes in the tumor cells. Our results reveal that *NHE1* antisense gene transfection can partly reverse the malignant behavior, resulting in intracellular acidification and induction of apoptosis of *SGC-7901* cells. These finding may provide a potential method for gastric carcinoma gene therapy in the future.

COMMENTS

Background

Gastric cancer is one of the most common malignant tumors in China, but the pathogenesis is unclear. Recent investigations have demonstrated that type 1 Na^+/H^+ exchanger (*NHE1*) mRNA expression is significantly increased in the carcinoma, relative to the occurrence and growth of tumors. Our previous studies have shown that significantly higher expression level of NHE1 protein in gastric carcinoma tissue compared to normal gastric tissue is closely associated with the genesis and progression of tumors. Therefore, the *NHE1* gene may be a good target for antisense gene therapy for gastric cancer. However, further studies are needed to determine whether antisense *NHE1* gene intervention can reduce type 1 Na^+/H^+ exchanger of the membranous ion exchange protein, and influence the biological behavior of the gastric carcinoma cells.

Research frontiers

Over expression of *NHE1* gene plays an important role in the regulation of pH_i in tumor cells, which is of important biological significance in the malignant growth of tumor cells. However, the role of NHE1 in the regulation of tumorigenic and metastatic properties of tumor cells remains unclear and it is important to determine the precise role of *NHE1*.

Innovations and breakthroughs

Our results reveal that *NHE1* antisense gene may significantly restrain the malignant behavior of human gastric carcinoma cells, result in intracellular acidification, suppress cell growth, induce cell apoptosis, and partially reverse the malignant phenotypes of *SGC-7901*. These findings suggest a potential role for human tumor gene therapy.

Applications

The present study may be helpful in determining a potential role for gastric carcinoma gene therapy in the future.

Terminology

The term pH_i means intracellular pH. The pH_i of tumor cells measured *in situ* is more alkaline than that of normal cells. This phenomenon of high pH_i with low extracellular pH (pH_e) is caused by the activation of *NHE1* in cell membrane and increased mRNA expression. *NHE1*, a type 1 Na^+/H^+ exchanger, is a transmembrane protein found in all eukaryotic cells. One of its functions is to reduce excess H^+ in the cytoplasm by means of $\text{Na}^+\text{-H}^+$ exchange, resulting in stable intracellular pH levels.

Peer review

This is an interesting article which examines the effect of inhibiting NHE1 on tumor survival. The manuscript is of interest and the data is good. It would be useful if NHE1 can be explained in more detail in the abstract.

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LIVER CANCER

Troglitazone, a peroxisome proliferator-activated receptor γ ligand, induces growth inhibition and apoptosis of HepG2 human liver cancer cells

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Zhou YM, Wen YH, Kang XY, Qian HH, Yang JM, Yin ZF. Troglitazone, a peroxisome proliferator-activated receptor γ ligand, induces growth inhibition and apoptosis of HepG2 human liver cancer cells. *World J Gastroenterol* 2008; 14(14): 2168-2173 Available from: URL: <http://www.wjgnet.com/1007-9327/14/2168.asp> DOI: <http://dx.doi.org/10.3748/wjg.14.2168>

Abstract

AIM: To examine the effect of troglitazone, a peroxisome proliferator-activated receptor γ (PPAR γ) ligand, on the proliferation and apoptosis of human liver cancer cells.

METHODS: Liver cancer cell line HepG2 was cultured and treated with troglitazone. Cell proliferation was detected by 3-(4-,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay; apoptosis was detected by flow cytometry and terminal deoxynucleotidyl transferase-mediated nick end labeling of DNA fragmentation sites (TUNEL) assay; and apoptosis-related protein was detected by immunocytochemistry and Western blotting.

RESULTS: Troglitazone inhibited growth and induced apoptosis of HepG2 cells in a dose-dependent manner, and induced activation of caspase-3 expression. Troglitazone not only drove apoptosis-inhibiting factor survivin to translocate incompletely from the nucleus to the cytoplasm, but also inhibited expression of survivin, while it did not affect expression of apoptosis-promoting factor Bax.

CONCLUSION: PPAR γ ligands inhibit growth and induce apoptosis of liver cancer cells, and may have applications for the prevention and treatment of liver cancer.

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Key words: Peroxisome proliferator-activated receptor γ ; Troglitazone; Liver neoplasms; Apoptosis

Peer reviewers: Frank A Anania, Professor, Emory University School of Medicine, Division of Division Digestive Diseases,

INTRODUCTION

Primary hepatocellular carcinoma (HCC) is the fifth most common malignant tumor worldwide, and causes more than 500 000 deaths annually^[1]. Dissemination of Hepatitis B virus (HBV) and Hepatitis C virus (HCV) causes the incidence of HCC to increase^[2]. Surgical resection is the treatment of choice for HCC, but only 10%-20% of HCC patients are resectable at the time of diagnosis^[3]. Liver transplantation is only indicated for early HCC (Milan criteria 1 tumor, < 5 cm in diameter, or up to three tumors, all < 3 cm in diameter), as far as outcome is concerned^[4]. In addition, recurrence remains high in patients who receive radical treatment^[5,6]. It is therefore urgent to seek a new approach for the treatment of HCC.

Peroxisome proliferator-activated receptor (PPAR) belongs to the nuclear hormone receptor superfamily^[7]. Three subtypes of PPAR (PPAR α , PPAR γ and PPAR δ) have been identified, among which PPAR γ has been studied most extensively. PPAR- γ exerts its effects by forming heterodimers with the 9-*cis*-retinoid X receptor (RXR) after ligand activation. The PPAR/RXR heterodimer acts as a regulatory transcription factor with peroxisome proliferator responsive element (PPRE) to regulate the expression of target genes which participate in the physiological and pathological processes of lipid metabolism, glucose metabolism, adipocyte differentiation, energy balance, inflammatory reactions and atherosclerosis^[8,9]. PPAR γ ligands are classified as natural and synthetic. The former include long-chain polyunsaturated fatty acid and eicosanoids, and the latter include thiazolidinediones (TZDs), the most commonly used drugs for diabetes, such as troglitazone, pioglitazone, rosiglitazone and LY171.833. Several recent studies have shown most human tumors

express PPAR γ , and there is evidence that PPAR γ ligands have anti-tumor activity^[10-20]. One of the important effects of these drugs is induction of apoptosis, although the exact mechanism remains elusive.

The present study used liver cancer cell line HepG2 that endogenously expresses PPAR γ as an experimental model^[15] to study the effects of PPAR γ ligand troglitazone on proliferation and apoptosis of liver cancer cells. It also analyzed the molecular mechanisms of these effects in an attempt to provide experimental clues for the use of PPAR γ ligands in the treatment of liver cancer.

MATERIALS AND METHODS

Chemicals

Troglitazone (Cayman Chemical Industry, USA) was dissolved in DMSO and then diluted to appropriate concentrations with culture medium. The final concentration of DMSO in the culture medium did not exceed 1 mL/L.

Cell culture

HepG2 cells were cultured in Dulbecco's modified Eagle's medium (GIBCO Laboratories, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS, GIBCO Laboratories), 100 U/mL penicillin, and 100 g/mL streptomycin. Cells were grown at 37°C in an atmosphere of 95% air and 5% CO₂.

MTT assay

Cell proliferation was detected by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (Sigma, St Louis, MO, USA) assay. When cells were cultured to the log phase, they were seeded on a 96-well plate (2×10^4 cells/100 μ L/well) for 24 h until confluence. Cells were divided into a control (DMSO) group and a troglitazone group. The concentration of troglitazone was 5, 10, 20, 40, 80 and 100 μ mol/L. After 120 h, 10 μ L MTT (5 g/L) was added and incubated at 37°C for 4 h. DMSO (75 μ L) was added to each well, which was oscillated for 10 min until the crystals were dissolved completely. Absorbance (A) was detected with an enzyme calibrator at 560 nm. Cell viability = (A of study group/A of control group) \times 100%. The experiment was repeated twice. There were six wells for each concentration.

Flow cytometry

Cells were seeded onto a six-well plate and allowed to grow to 40% confluence. After treatment with or without the drug, both floating and adherent cells were collected. Cells were suspended and then fixed with ice-cold 70% alcohol at -20°C, followed by washing and staining with 50 μ g/mL propidium iodide in the presence of 100 μ g/mL ribonuclease A for 30 min at 37°C in the dark. DNA content was analyzed by flow cytometry using Cell Quest software (Becton Dickinson and Beckman-Coulter, San Jose, CA, USA).

TUNEL assay

HepG2 cells were grown on poly-L-lysine-coated slides in a six-well plate. After treatment with or without the drug, the slides were gently washed three times in 0.1 mol/L PBS

(pH 7.4), fixed with 80% ice-cold ethanol, and immediately transferred to a freezer until use. To study apoptosis of cultured cells, terminal deoxynucleotidyl transferase-mediated nick end labeling of DNA fragmentation sites (TUNEL) assay was performed using Fluorescein FragEL DNA Fragmentation Detection Kit (Oncogene Research Products, San Diego, CA, USA) according to the manufacturer's instructions. Ten high-power areas with even distribution of positive cells were used for calculation of the percentage of apoptotic cells under a fluorescence microscope.

Immunocytochemistry

HepG2 cells were grown on poly-L-lysine-coated slides in a six-well plate. After treatment with the drug, the slides were washed twice with PBS and fixed in 4% paraformaldehyde for 30 min at room temperature. Immunostaining was performed using the streptavidin-biotin complex method with UltraSensitive S-P Kit (Fuzhou Maxim Biotech, Fuzhou, Fujian, China). The slides were pretreated first with 0.3% hydrogen peroxide in PBS for 10 min to inactivate endogenous peroxidase, and then microwave antigen retrieval was performed with 0.01 mol/L citrate buffer at pH 6.0 for 20 min, followed by incubation with polyclonal rabbit anti-human antibody against the active caspase-3 (1:25; R&D Systems, Wiesbaden, Germany), rabbit anti-human survivin polyclonal antibody (1:50; Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 4°C overnight, biotinylated secondary antibody for 30 min, and peroxidase-labeled streptavidin for 30 min. Color reaction was developed with 3, 3'-diaminobenzidine as a chromogen. Finally, the slides were counterstained with hematoxylin, and dehydrated through graded alcohol. For negative controls, slides were processed as above but treated with PBS instead of the primary antibody.

Western blotting

Troglitazone-treated HepG2 cells were prepared in a cell-lysis solution. The protein concentration was determined by BCA Protein assay kit (Perbio Science Deutschland, Germany). Protein samples (50 μ g) were separated by SDS-PAGE and transferred to nitrocellulose membranes, to which rabbit anti-human survivin polyclonal antibody (1:1000), mouse anti-human Bax antibody (1:1000), mouse anti-human β -actin antibody (1:1000), and horseradish-peroxidase-labeled secondary antibody (all from Santa Cruz Biotechnology) were added. The signals were detected by enhanced chemiluminescence according to the protocol supplied with the kit.

Statistical analysis

Data were expressed as mean \pm SD. Statistical correlation of data was checked for significance by ANOVA and Student's *t* test. Differences with *P* < 0.05 were considered significant. These analyses were performed using SPSS 11.0 software (SPSS, Chicago, IL, USA).

RESULTS

Troglitazone inhibits cell growth

The MTT assay showed that cell viability was $96.8\% \pm 1.22\%$,

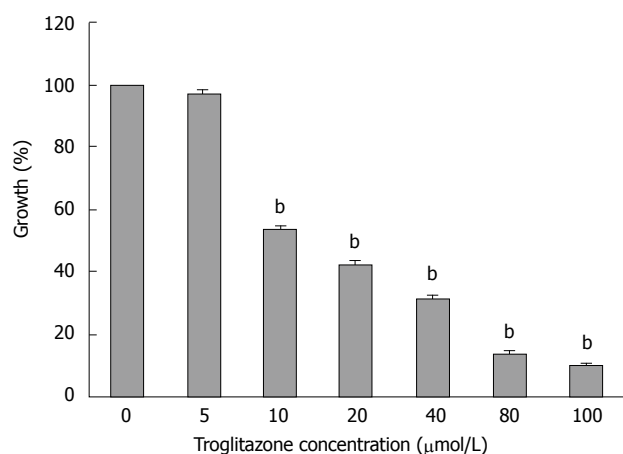


Figure 1 Effect of troglitazone on growth of HepG2 cells. Cells were incubated with different concentrations of troglitazone for 120 h. Troglitazone inhibited growth of HepG2 cells in a dose-dependent manner, as demonstrated by MTT assay. Data represent the mean \pm SD from six wells. ^b $P < 0.01$ vs corresponding control group (unpaired Student's t test).

53.4% \pm 1.2%, 42.3% \pm 1.2%, 31.4% \pm 1%, 13.6% \pm 0.8% and 9.6% \pm 0.7% at 5, 10, 20, 40, 80 and 100 μ mol/L troglitazone for 120 h, respectively (Figure 1), which indicated that troglitazone inhibited the growth of liver cells in a dose-dependent manner.

Troglitazone induces apoptosis

HepG2 cells were treated with troglitazone (20 and 30 μ mol/L) for 24 h, and phase-contrast microscopy revealed that some cells became round, blunt and smaller in size, accompanied by karyopyknosis; light refraction was increased; and cells became detached and suspended in the medium, especially with 30 μ mol/L troglitazone. In the control group, cells were regular in morphology and grew fully in patches and confluent, rarely sloughing off (Figure 2).

Flow cytometry showed that after treatment of HepG2 cells with 20 and 30 μ mol/L troglitazone for 24 h, the percentage of sub-G₀/G₁ peak apoptotic cells was 25.5% \pm 1.1% and 43.9% \pm 1.7%, respectively. In the untreated controls, none of the cells was found in the sub-G₀/G₁ peak (Figure 3). TUNEL staining also showed that treatment with troglitazone for 24 h caused apoptosis of HepG2 cells. The apoptotic rate increased with concentration of troglitazone: 22.4% \pm 1.2% for 20 μ mol/L, and 38.6% \pm 0.8% for 30 μ mol/L troglitazone. In the control group, there were only background cells, without the presence of TUNEL positive cells (Figure 4, $P < 0.001$).

Troglitazone induces activation of caspase-3 expression

The caspase family plays a very important role in mediating apoptosis, among which caspase-3 is the key executive molecule, and its activated form has multiple functions in signal transmission of apoptosis^[21,22]. Troglitazone activated caspase-3 expression. Positive staining was signified by a yellow-brown color, present in smaller cells whose plasma was condensed. After treatment of HepG2 cells with 20 and 30 μ mol/L troglitazone, the positive cell rate was 21.2% \pm 1.4% and 35.8% \pm 2.4%, respectively (Figure 5). No positive cells were found in the controls.

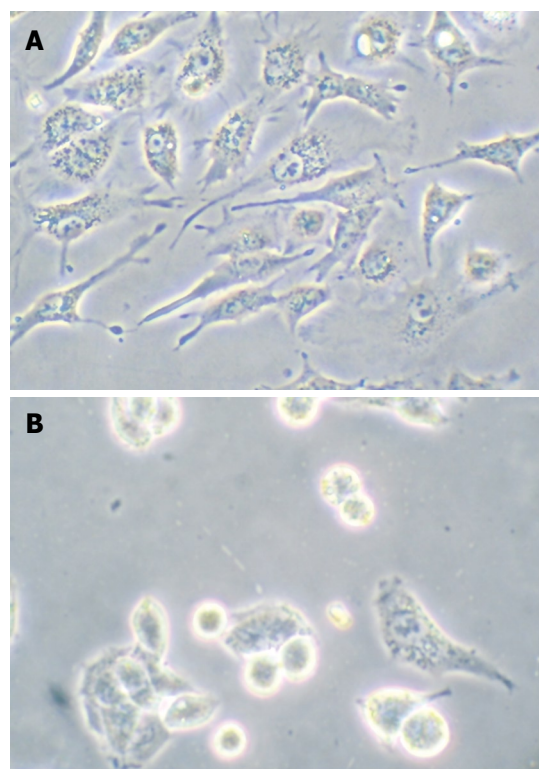


Figure 2 Morphological changes were examined by phase-contrast microscopy. A: Controls; B: 30 μ mol/L troglitazone.

Troglitazone drives survivin protein to translocate incompletely from the nucleus to the cytoplasm and inhibits its expression

Immunocytochemical staining showed that apoptosis-inhibiting factor survivin was mainly expressed in the nucleus and weakly in the cytoplasm of untreated HepG2 cells. After treatment with 20 and 30 μ mol/L troglitazone for 24 h, survivin translocated partly from the nucleus to the cytoplasm, and the intensity of its expression decreased markedly. Also, apoptotic cells were seen in the treatment group, and presented with a smaller cell size and condensed cytoplasm (Figure 6). Western blotting revealed that, in HepG2 cells treated with troglitazone for 24 h, expression of HepG2 survivin was inhibited in a dose-dependent manner, but the treatment did not significantly affect expression of apoptosis-promoting factor Bax (Figure 7).

DISCUSSION

Apoptosis is a normal phenomenon in the process of embryonic growth of all organisms and human development. Disturbance of this process is associated with the development of many severe diseases and disorders including tumors. Selective induction of tumor-cell apoptosis may become the basic strategy in the treatment of malignant tumors. Most of the available chemotherapy drugs work through destroying tumor cells by inducing apoptosis^[23]. In our study, the MTT assay showed troglitazone inhibited growth of liver cancer cells in a dose-dependent manner. To determine whether the inhibitory effect of troglitazone on the proliferation of liver cancer cells was associated with induction of apoptosis, flow cytometry and TUNEL

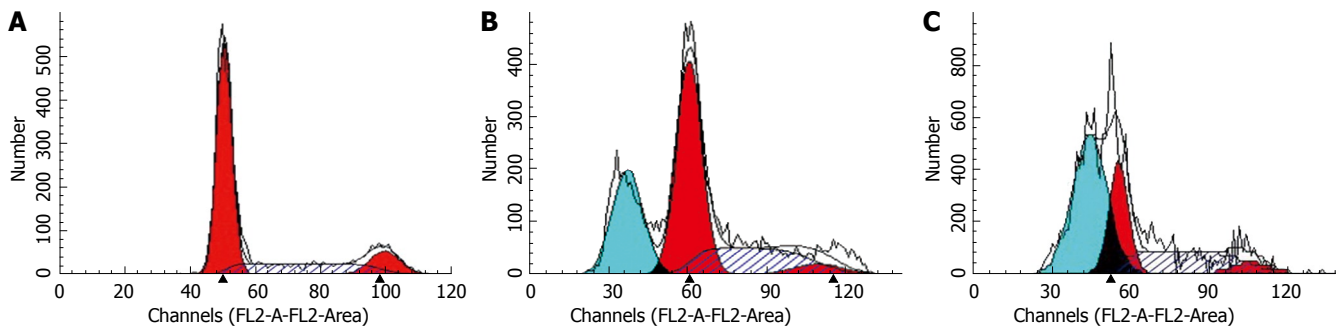


Figure 3 Cell cycle analysis by flow cytometry. **A:** Control cells; **B:** 20 $\mu\text{mol/L}$ troglitazone; **C:** 30 $\mu\text{mol/L}$ troglitazone.

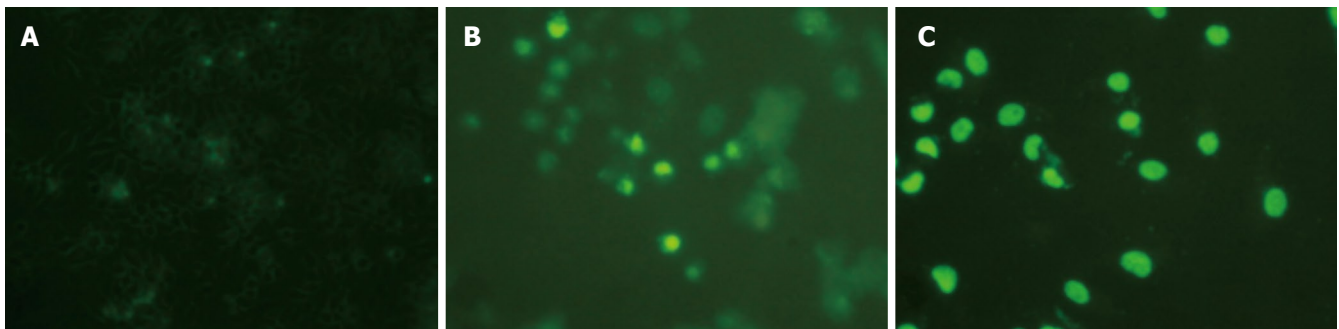


Figure 4 Troglitazone significantly increased the number of TUNEL-positive cells in a dose-dependent manner. **A:** Control cells; **B:** 20 $\mu\text{mol/L}$ troglitazone; **C:** 30 $\mu\text{mol/L}$ troglitazone.

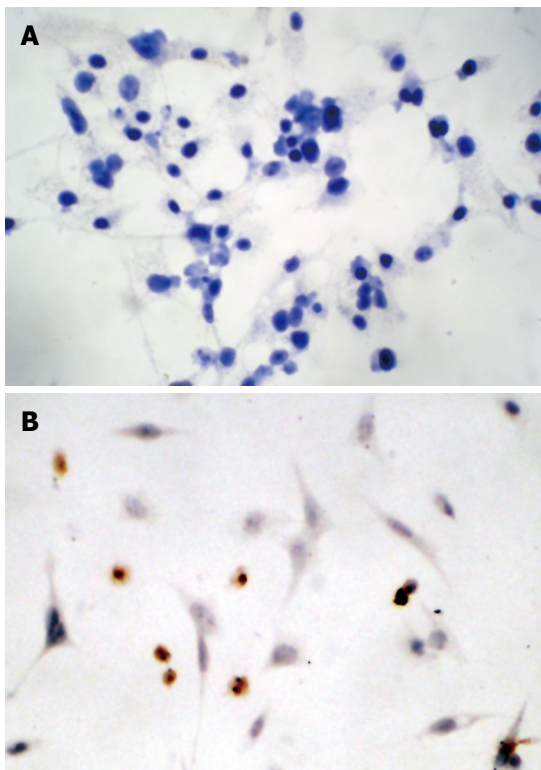


Figure 5 Immunocytochemistry showed that caspase-3 was activated. The shrunken cells were positively stained for activated caspase-3. No positive cell was detectable in the control group (**A:** original magnification, $\times 200$). Whereas large numbers of positive cells labeled for activated caspase-3 were observed in HepG2 cells treated with 30 $\mu\text{mol/L}$ troglitazone for 24 h (**B:** original magnification, $\times 400$).

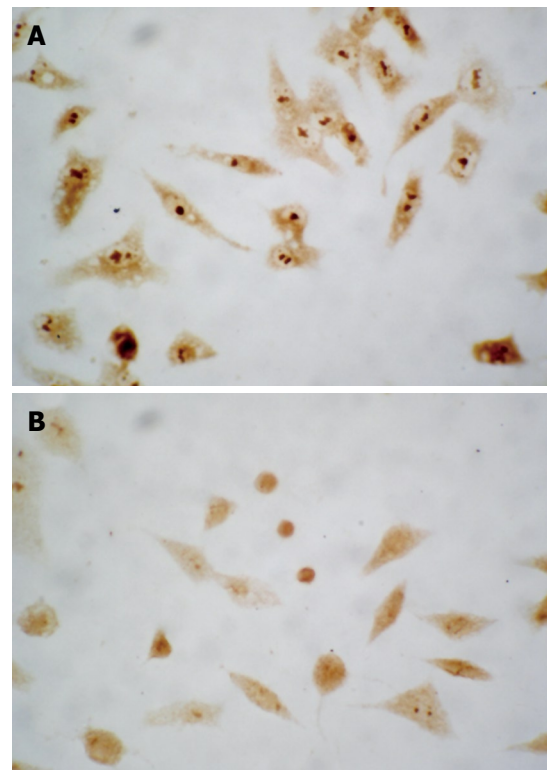


Figure 6 Survivin was present predominantly in the nucleus in untreated cells (**A:** original magnification, $\times 400$) as demonstrated by immunocytochemistry. After exposure of HepG2 cells to 30 $\mu\text{mol/L}$ troglitazone for 24 h, survivin incompletely translocated from the nucleus to the cytoplasm (**B:** original magnification, $\times 400$).

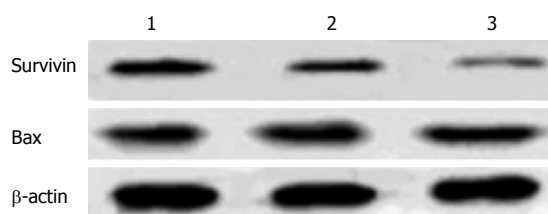


Figure 7 Effect of troglitazone on the expression of Bax and survivin. Lane 1: Control cells; lane 2: 20 $\mu\text{mol/L}$ troglitazone; lane 3: 30 $\mu\text{mol/L}$ troglitazone.

staining were conducted. The results confirmed troglitazone induced apoptosis of HepG2 cells in a dose-dependent manner. Thus, clarifying the apoptosis-related gene protein that is associated with troglitazone-induced apoptosis is of primary importance in explaining the mechanism of action of troglitazone.

Caspase is a group of cysteine hydrolytic proteinases that are able to specifically cleave the peptide chain behind the residue base of the target protein aspartate. It is the key molecule in the regulation of apoptosis. Caspase can trigger a cascade reaction under the control of apoptosis signals. According to their method of entering the apoptotic site, caspases are classified as initiator and effector caspases^[21], among which, caspase-3 is the most important terminal effector caspase in apoptosis, and plays an irreplaceable role. After initiation of the apoptotic process, caspase-3 transforms from the zymogen form to the activated form and functions by hydrolyzing proteins essential for survival of many kinds of cells^[22].

Survivin is a member of the inhibitor of apoptosis (IAP) family of negative regulators of programmed cell death, and is undetectable in normal adult tissue, but is abundantly expressed in fetal tissue and a variety of human cancers including HCC. High levels of survivin in tumors are frequently correlated with malignant parameters, which suggest a role in tumorigenesis^[24-26]. Survivin can inhibit terminal effector caspase-3 and caspase-7, and interfere with caspase-9 activity and processing. Therefore, approaches designed to counteract antiapoptotic activity may inhibit tumor growth^[27].

There has long been controversy over the subcellular location of survivin. Some researchers have found survivin expression is located in the nucleus in HCC, esophageal squamous cell carcinoma, ovarian carcinoma, mantle cell lymphoma, cholangiocarcinoma, and non-small cell lung cancer, and is associated with poor prognosis. Other researchers have pointed out that survivin expression is located in the nucleus in gastric cancer, bladder mucosa and transitional cell carcinoma, and breast cancer, and is a predictor of good prognosis^[28]. The results of our immunohistochemical staining confirmed that survivin was expressed mainly in the nucleus of HepG2 cells, and weakly in the cytoplasm. Fortugno *et al*^[29] have found two survivin pools using immunofluorescence, one in the cytoplasm and the other in the nucleus. Suzuki *et al*^[30] have found survivin protein can translocate from the cytoplasm to the nucleus. Here, it forms a complex with CDK4, which enables p21 to combine with a precursor released from the CDK4 complex and mitochondrial caspase-3, and inhibits

apoptosis by inhibiting the activity of caspase-3. The results of our study showed troglitazone induced apoptosis in a dose-dependent manner, during which survivin translocated incompletely from the nucleus to the cytoplasm, with a decrease in the intensity of expression; at the same time caspase-3 expression was activated. We presume troglitazone dissociated the survivin/CDK4 complex by inducing survivin expression and driving survivin to translocate incompletely from the nucleus to the cytoplasm. This forced p21 to form a complex with CDK4 by dissociating the combination of p21 and the precursor of mitochondrial caspase-3 and activating caspase-3. This led to cleavage of the corresponding nuclear and cytoplasmic substrates and finally caused apoptosis.

TZD induces massive apoptosis in renal cancer cells, with increased Bax expression and decreased Bcl-2 expression^[18]. However, troglitazone significantly increases the expression of c-myc mRNA, but has no effect on expression of Bcl-2 and Bax in thyroid carcinoma cells^[13]. In the current study, we also found Bax may not participate in troglitazone-induced apoptosis, suggesting that the mechanisms by which the PPAR γ ligands cause tumor cell apoptosis are different depending on the type of cancer.

In conclusion, PPAR γ ligands have the effect of inhibiting growth and inducing the apoptosis of liver cancer cells, and may have applications for the prevention and treatment of liver cancer.

COMMENTS

Background

Peroxisome proliferator-activated receptor (PPAR) belongs to the nuclear hormone receptor superfamily. Three subtypes of PPAR (PPAR α , PPAR γ and PPAR δ) have been identified, among which PPAR γ has been studied most extensively. Recent data have shown that ligands for PPAR γ exhibit growth-inhibitory effects on many types of human cancer. One of the important effects of these drugs is inducing apoptosis, although the exact mechanism remains elusive.

Research frontiers

We used liver cancer cell line HepG2 that endogenously expresses PPAR γ as an experimental model to study the effects of PPAR γ ligand troglitazone on the proliferation and apoptosis of liver cancer cells, and analyzed the molecular mechanisms of these effects.

Innovations and breakthroughs

Troglitazone inhibited growth and induced apoptosis of HepG2 cells in a dose-dependent manner, and induced activation of caspase-3 expression. Troglitazone not only drove apoptosis-inhibiting factor survivin to translocate incompletely from the nucleus to the cytoplasm, but also inhibited expression of survivin.

Applications

PPAR γ ligand has the effect of inhibiting growth and inducing apoptosis of liver cancer cells, and may have potential as a drug against liver cancer.

Peer review

This study should arouse interest in readers. It provides important information for the investigation of the anti-cancer mechanism of troglitazone and other PPAR γ ligands, and for the development of anti-cancer therapy using PPAR γ ligands.

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BASIC RESEARCH

Detection of apoptosis induced by new type gosling viral enteritis virus *in vitro* through fluorescein annexin V-FITC/PI double labeling

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PI positive) through flow cytometry and fluorescence microscope. The percentage of apoptotic cells increased with the incubation time and reached a maximum at 120 h after infection, while the percentage of non-apoptotic cells decreased.

CONCLUSION: NGVEV can induce the infected DEF cells to undergo apoptosis and the apoptosis occurs prior to necrosis.

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Key words: Gosling viral enteritis; New type; Virus; Duck embryo fibroblasts; Apoptosis; Fluorescein annexin V-FITC/PI

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Abstract

AIM: To achieve a better understanding of the pathogenesis of new type gosling viral enteritis virus (NGVEV) and the relationship between NGVEV and host cells.

METHODS: The apoptosis of duck embryo fibroblasts (DEF) induced by NGVEV was investigated by fluorescence-activated cell sorter (FACS) and fluorescence microscope after the cells were stained with Annexin V-FITC and propidium iodide (PI).

RESULTS: By staining cells with a combination of fluorescein annexin V-FITC and PI, it is possible to distinguish and quantitatively analyze non-apoptotic cells (Annexin V-FITC negative/PI negative), early apoptotic cells (Annexin V-FITC positive/PI negative), late apoptotic/necrotic cells (Annexin V-FITC positive/PI positive) and dead cells (Annexin V-FITC negative/

INTRODUCTION

In order to eliminate the redundant, damaged, or infected cells, metazoan organisms evolve the cell suicide mechanism termed apoptosis^[1]. Apoptosis is a physiological process defined by a number of distinct morphological features and biochemical processes^[2,3], which distinguish from necrosis^[4,5]. Apoptosis is recognized as an important process in different biological systems, including embryonic development, cell turnover, and immune response against tumorigenic or virus-infected cells^[6-8]. An increasing number of viruses or viral gene products were reported to induce apoptosis both *in vitro* and *in vivo*^[9-16].

The new type gosling viral enteritis (NGVE) is a new infectious disease and firstly recognized by Cheng *et al*, and it was observed in goslings less than 30 d of age in various areas of Sichuan Province^[17,18]. The mortality from acute NGVE is high, and it is clinically characterized by respiratory, digestive, and neurological symptoms and

sudden death^[17-19]. Catarrhal hemorrhagic fibrinonecrotic enteritis of the small intestine and coagulative embolus in the lower middle part of the intestine are the typical pathological changes of the NGVE in infected goslings^[18]. NGVE virus was recognized as an adenovirus, which was round or oval, and characteristic icosahedral in shape, containing double-stranded DNA genome and fifteen structural proteins^[18-20]. There are many researches on the histopathology, epizootiology, clinical signs, diagnoses, and immunity of the NGVE^[17-24]. Interestingly, the apoptosis induced by NGVE virus infection is poorly documented.

In the early stage of apoptosis, which occurs at the cell surface, one of these plasma membrane alterations is the translocation of phosphatidylserine (PS) from the inner side of the plasma membrane to the outer layer, by which PS becomes exposed at the external surface of the cell^[25-27]. Annexin V-FITC is a phospholipid-binding protein with a high affinity for PS, which can be used as a sensitive probe for PS exposure to the cell membrane^[28-30]. However, it has also been reported that it binds to the inner face of the plasma membrane that has lost its integrity during the late stage of apoptosis, also known as secondary necrosis^[31]. During the early apoptosis, the cells become reactive with annexin V-FITC after the onset of chromatin condensation, but prior to the loss of the plasma membrane ability to exclude PI^[32]. Hence, necrotic cells are both stained by annexin V-FITC and PI, whereas early apoptotic cells are only stained by annexin V-FITC. Double staining of the infected DEF cells with annexin V-FITC and PI in this research could distinguish apoptotic cells from necrotic cells^[33]. In this way, live, early apoptotic, late apoptotic/necrotic and dead cells can be discriminated on the basis of a double-labeling for annexin V-FITC and PI, and analyzed by either flow cytometry or fluorescence microscopy^[34,35].

MATERIALS AND METHODS

Primary duck embryo fibroblast (DEF) and viral strain

DEF cells were prepared using 11 to 13-d-old embryonated specific pathogen-free (SPF) eggs and propagated in minimal essential medium (MEM; Gibco) containing 100 mL/L new born calf serum (NBCS; Hyclone), 2.2 g/L NaHCO₃, 100 U/mL penicillin/streptomycin (Gibco).

The NGVEV-CN strain with a high virulence field was provided by the Avian Diseases Research Centre of the Sichuan Agricultural University. The initial strain (adapted for cell culture growth) was isolated from a natural NGVE virus infection and the SPF gosling was then artificially infected, and the virus was serially passaged in 10-day-old SPF embryo eggs. The allantoic fluid was harvested and adapted to the monolayer DEF cells.

Experimental NGVE virus infection of DEF

The monolayer DEF cells were washed twice with phosphate buffered saline solution (PBS; 0.15 mol/L, pH 7.2) and subsequently exposed to stock NGVEV-CN on a shaker at 37.5°C for 2 h. Stock virus was harvested from infected DEF when 75% cytopathic effects (CPEs) were observed. After inoculation with NGVEV-CN, cells were

Table 1 Apoptotic rate of DEF cells detected through annexin V-FITC/PI staining and analyzed by FACS

Infection time (h)	Early apoptotic cells (%) (annexin V-FITC ⁺ /PI ⁻)		Advanced apoptotic cells (%) (annexin V-FITC ⁺ /PI ⁺)	
	Mock infected	NGVEV infected	Mock infected	NGVEV infected
24	3.6	4.3	0.3	0.3
48	3.9	8.8	0.8	1.3
72	4.6	21.5	1.7	1.9
96	5.2	30.8	3.4	7.8
120	6.1	35.3	4.5	13.9
144	7.2	33.7	5.1	17.7

cultured at 37°C in a humidified atmosphere of 5% CO₂ in MEM supplemented with penicillin/streptomycin and 20 mL/L NBCS. Mock-infected cells were processed in the same way except that the virus was excluded.

Annexin V-FITC/PI stained fluorescence-activated cell sorter (FACS)

At 24, 48, 72, 96, 120 and 144 h after infection (p.i.), 3 infected and mock-infected cells were harvested through trypsinization, and washed twice with cold PBS (0.15 mol/L, pH 7.2). The cells were centrifuged at 3000 r/min for 5 min, then the supernatant was discarded and the pellet was resuspended in 1 × binding buffer at a density of 1.0 × 10⁵-1.0 × 10⁶ cells per mL. One hundred µL of the sample solution was transferred to a 5 mL culture tube, and incubated with 5 µL of FITC-conjugated annexin V (Pharmingen) and 5 µL of PI (Pharmingen) for 15 min at room temperature in the dark. Four hundred µL of 1 × binding buffer was added to each sample tube, and the samples were analyzed by FACS (Becton Dickinson) using Cell Quest Research Software (Becton Dickinson).

Annexin V-FITC/PI stained fluorescence microscopy

The annexin V-FITC/PI staining procedure of the sample was adopted as above except that 1.0 × 10⁶ cells/mL were centrifuged onto glass slides and studied under fluorescence microscope (Nikon 80i).

RESULTS

Annexin V-FITC/PI stained FACS

By staining cells with annexin V-FITC and PI, FACS was used to distinguish and quantitatively determine the percentage of dead, viable, apoptotic and necrotic cells after NGVE virus infection (Figure 1 and Table 1). At 72 h p.i., the percentage of apoptotic cells increased from 4.6% in the mock-infected control culture to 21.5% (Figure 2). The percentage of early apoptotic cells increased with incubated time until 120 h p.i. reaching the maximum 35.3%, and the proportion of the late apoptotic/necrotic cells increased from 0.3% to 17.7% (Table 1). A high level of the early apoptosis was detected from 72 h p.i. and high level of the late apoptosis/necrosis was detected after 96 h p.i., while the basal level of apoptosis and necrosis was shown in the mock-infected controls (Table 1).

Annexin V-FITC/PI stained fluorescence microscopy

When examined under fluorescence microscopy, different

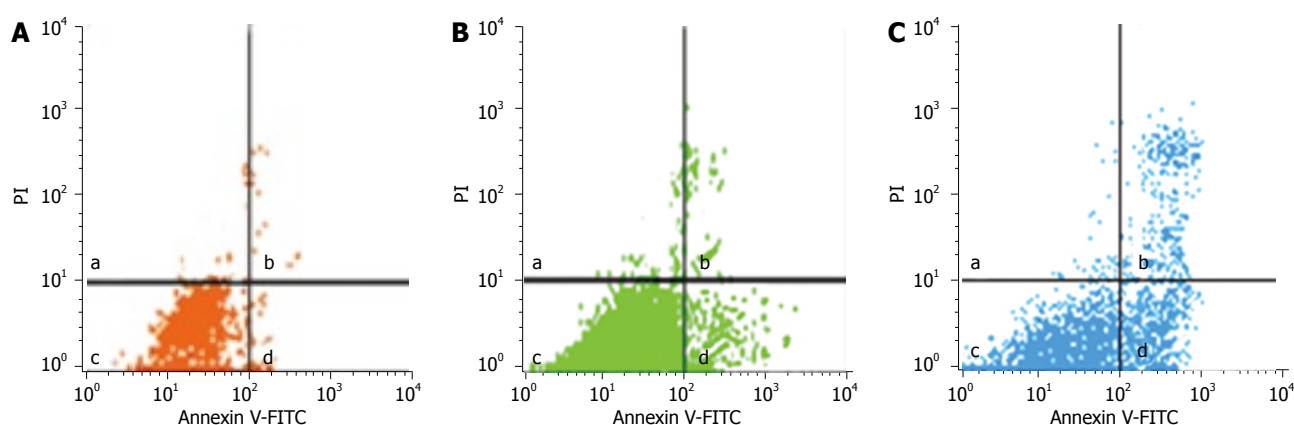


Figure 1 NGVE virus infected DEF cells analyzed by FACS, stained with annexin V-FITC/PI. (A-C) display the results of the cells at 48, 96 and 144 h after NGVE virus infection. The proportion of non-apoptotic cells (c: Annexin V-FITC⁻/PI⁻), early apoptotic cells (d: Annexin V-FITC⁺/PI⁻), late apoptotic/necrotic cells (b: Annexin V-FITC⁺/PI⁺) and dead cells (a: Annexin V-FITC⁻/PI⁺).

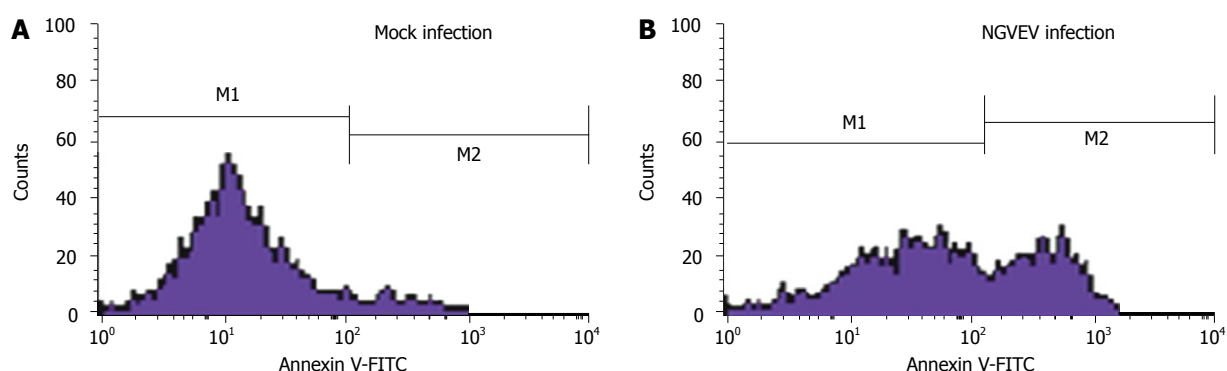


Figure 2 Flow cytometry of apoptotic DEF cells as assessed by annexin V-FITC fluorescent intensity. DEF cells are mock infected (A) and infected with NGVE virus (B). Cells harvested at 72 h p.i., and subsequently stained with annexin V-FITC/PI. One million cells are analyzed by flow cytometry, data are presented as fluorescent intensity units of annexin V-FITC (abscissa) and number of counts cells (ordinate). The M1 and M2 gates demarcate annexin V-FITC negative populations (non-apoptotic cells) and positive (apoptotic cells) populations.

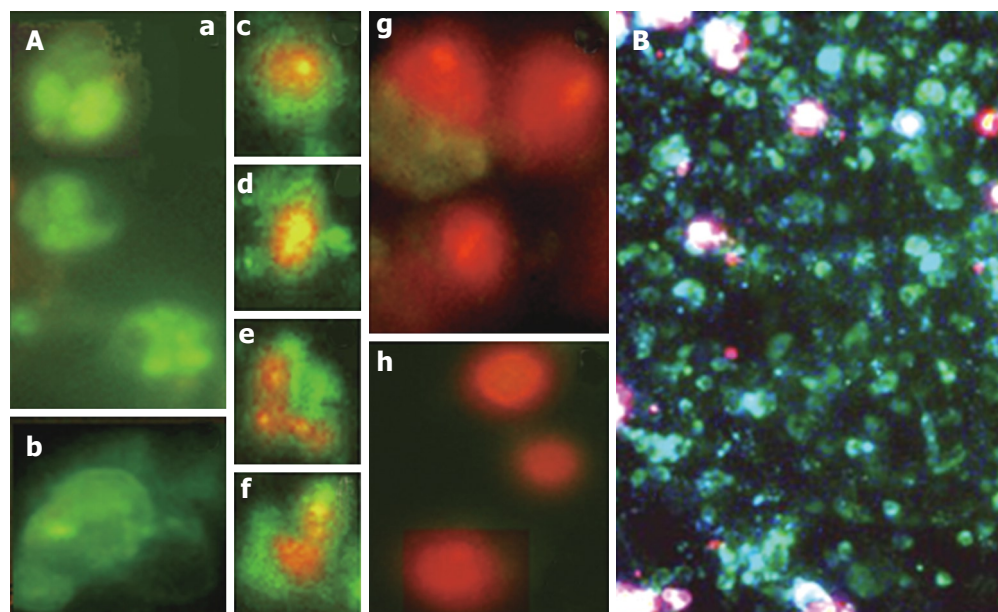


Figure 3 Apoptotic DEF cells induced by NGVE virus infection stained with Annexin V-FITC/PI and observed under fluorescence microscope. The samples are analyzed for green fluorescence (FITC) and red fluorescence (PI). **A**: Different labeling patterns of the NGVE virus infected cells: early apoptotic cells, annexin V-FITC positive and PI negative (a and b); necrotic or late apoptotic cells, both annexin V-FITC and PI positive (c-f); dead cells, annexin V-FITC negative and PI positive (g and h); **B**: 72 h p.i., the early and late apoptotic cells.

labeling patterns in this assay enabled us to identify different cell populations: live cells (Annexin V-FITC negative/PI negative), early apoptotic cells (the intactness

of the cell membrane, affinity for annexin V-FITC and devoid of PI staining) (Figure 3A a, b, 3B), late apoptotic/necrotic cells (the cell membrane loses its integrity,

the cell becomes both annexin V-FITC and PI staining) (Figure 3A c-f, 3B) and dead cells (Annexin V-FITC negative/PI positive) (Figure 3A g, h, 3B).

DISCUSSION

Modulation of apoptosis is a common feature of infection by animal viruses and it also contributes to the pathogenesis process^[36]. A variety of animal viruses have been identified to induce apoptosis in cultured cells^[12,14-16], which contained adenovirus. Early in 1968, Takemori^[37] found that *cyt* mutants of human adenovirus could provoke more violent CPEs. Ezoe^[38] further proved that it could also induce the DNA degradation in infected cells. Rautenschlein^[39,40] respectively reported that the hemorrhagic enteritis virus (HEV) (fowl adenovirus) could induce B cells and spleen cells undergoing apoptosis. This research indicated that NGVE virus recognized as an adenovirus^[17,19,20] could induce DEF undergoing apoptosis, which has never been reported before.

FACS is frequently used to monitor early apoptosis^[26-29], which should always be confirmed by the inspection of cells under electron or fluorescence microscope. Annexin V-FITC positive cells were first observed in NGVEV-infected DEF cells at 72 h p.i. under fluorescence microscopy, while it can be detected early from 24 h p.i. through FACS. The small number of apoptotic cells presented in mock-infected controls, which may be attributed to physiological cell death *in vitro*. The cells stained by annexin V-FITC alone obviously increased from 72 h p.i., indicating the induction of apoptosis rather than necrosis due to NGVE virus infection. The cells that stained positive for both annexin V-FITC and PI were increased from 96 h p.i. indicated the end stage of apoptosis or necrosis, which also suggested that apoptosis occurs prior to necrosis. This may be due to the fact that apoptosis makes many cell remnants undisturbed *in vitro*, which can be removed by phagocytes *in vivo*. The apoptotic cell debris interfered with the adjacent normal cells, leading to the necrosis. Furthermore, the lysis that eventually occurred at the end of apoptosis, which had essentially the same membrane permeability that occurred in necrosis. Further experiments are needed for a definite the intracellular events that trigger the apoptotic response during NGVE virus infection.

Recent studies demonstrate that the CPEs caused by virus infection *in vitro* is mediated by apoptosis^[41-43]. Our previous research had revealed that the CPEs became obvious after 72 h p.i.^[24] and TCID₅₀ reached a maximum at 120 h p.i., which was consistent to the results of this research: apoptotic cells obviously increased from 72 h p.i. and the apoptotic peak reached at 120 h p.i.. Therefore, it seems likely that apoptosis is related to CPEs during NGVE virus infection.

Virus-induced apoptosis is a complex and important aspect of the pathogenesis of viral infection^[44-46]. In fact, in the case of virus-infected cells, the induction of cell death can reduce viral spread in the host by early killing of infected cells. In the case of virus itself, apoptosis facilitates persistent viral infection in host cells and is convenient for viral dissemination^[47-49]. Quantitative assay

of the apoptosis in the present study indicated that the apoptosis was largely induced in the late phase of NGVE virus infection. During late NGVE virus infection, the virus almost completes its replication, therefore, the apoptosis provided a means for releasing the virus particles into the extracellular space without initiating a concomitant host response. It is assumed that NGVE virus induction of apoptosis may be an important mechanism of the efficient dissemination of progeny and the suicide of virally infected cells through apoptosis can limit infection, affording the host organism a certain degree of protection.

Many questions regarding NGVE virus-induced apoptosis remain unanswered, and future studies should be carried out.

COMMENTS

Background

New type gosling viral enteritis (NGVE) is a new infectious disease and it is observed in goslings aged less than 30 d. The typical pathological changes of the NGVE in infected goslings are catarrhal hemorrhagic fibrinonecrotic enteritis of the small intestine and coagulative embolus in the lower middle part of the intestine. NGVE virus is recognized as an adenovirus and many reports indicated that adenovirus could induce apoptosis both *in vitro* and *in vivo*. To date, whether the NGVE virus could trigger the host cells to undergo apoptosis has not been reported.

Research frontiers

A number of viruses or viral gene products have been reported to induce apoptosis both *in vitro* and *in vivo*. Modulation of apoptosis is a common feature of infection by animal viruses and it was proved to contribute to the pathogenesis process. Scant information has been available so far for NGVE, especially in its etiology and pathogenesis. The apoptosis detected in this research during NGVE virus infection may be responsible for its pathogenesis.

Innovations and breakthroughs

The authors of this paper have indicated that, for the first time, NGVE virus could induce the apoptosis of host cells *in vitro* and the apoptosis occurs prior to necrosis. The combined use of the fluorochrome labeled with fluorescence-activated cell sorter and fluorescence microscope for apoptosis detection can provide a rapid, quantitative and objective assay of cell viability, which may be applied for enumeration of apoptotic or necrotic cells.

Applications

This work succeeded in a better understanding of pathogenesis process during NGVE virus infection.

Terminology

Apoptosis: also named as programmed cell death (PCD), is the process whereby individual cells of multicellular organisms undergo systematic self-destruction in response to a wide variety of stimuli. Apoptosis is a genetically controlled preprogrammed event which eliminates cell development when they have become redundant, or functions as an emergency response after radiation damage, viral infection, or aberrant growth induced by the activation of oncogenes.

Peer review

This is a very interesting study. The authors demonstrated that NGVE virus induces the apoptosis of infected DEF cells and the apoptosis occurs prior to necrosis. The study is well designed, and the analysis is reasonable.

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Pharmacokinetics and tissue distribution of intraperitoneal 5-fluorouracil with a novel carrier solution in rats

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Abstract

AIM: To compare the pharmacokinetics and tissue distribution of 5-fluorouracil administered intraperitoneally with two isotonic carrier solutions: HAES-steri (neotype 6% hydroxyethyl starch), a novel carrier solution with middle molecular weight and physiologic saline (0.9% sodium chloride solution), a traditional carrier solution for intraperitoneal chemotherapy, in rats.

METHODS: A total of 60 Sprague Dawley rats were randomized into groups according to the carrier solution administered. Each group was further randomized according to the intraperitoneal dwell period (1, 3, 6, 12, 18 and 24 h). At the end of the procedure the rats were killed, the peritoneal fluid was withdrawn completely and quantitated. Drug concentrations in peritoneal fluid, plasma, and tissues were determined by high-performance liquid chromatography.

RESULTS: The mean volumes remaining in the peritoneal cavity were significantly higher with HAES-steri than those with physiologic saline at 1, 6, 12, 18, and 24 h ($P = 0.047, 0.009, 0.005, 0.005$ and 0.005 respectively, the percentages of remaining peritoneal fluid volume were 89.9 ± 5.6 vs 83.4 ± 4.9 , 79.9 ± 2.8 vs 56.2 ± 15.7 , 46.8 ± 5.5 vs 24.7 ± 9.7 , 23.0 ± 2.8 vs 0.0 ± 0.0 and 4.2 ± 1.7 vs 0.0 ± 0.0 respectively). Mean concentrations in peritoneal fluid were significantly higher with HAES-steri than those with physiologic saline at 3, 12 and 18 h ($P = 0.009, 0.009$ and 0.005 respectively, the concentrations were 139.2768 ± 28.2317 mg/L vs

mg/L, 11.5427 ± 3.0976 mg/L vs 0.0000 ± 0.0000 mg/L and 4.7724 ± 1.0936 mg/L vs 0.0000 ± 0.0000 mg/L respectively). Mean plasma 5-fluorouracil concentrations in portal vein were significantly higher with HAES-steri at 3, 12, 18 and 24 h ($P = 0.009, 0.034, 0.005$ and 0.019 respectively, the concentrations were 3.3572 ± 0.8128 mg/L vs 0.8794 ± 0.2394 mg/L, 0.6203 ± 0.9935 mg/L vs 0.0112 ± 0.0250 mg/L, 0.3725 ± 0.3871 mg/L vs 0.0000 ± 0.0000 mg/L, and 0.2469 ± 0.1457 mg/L vs 0.0000 ± 0.0000 mg/L respectively), but significantly lower at 1 h ($P = 0.009$, the concentrations were 4.1957 ± 0.6952 mg/L vs 7.7406 ± 1.2377 mg/L). There were no significant differences in the plasma 5-fluorouracil in inferior caval vein at each time-point. 5-fluorouracil concentrations were significantly greater with HAES-steri at 18 h in gastric tissue ($P = 0.016$, the concentrations were 0.9486 ± 0.8173 mg/L vs 0.30392 ± 0.0316 mg/L), at 18 h in colon ($P = 0.009$, the concentrations were 0.1730 ± 0.0446 mg/L vs 0.0626 ± 0.0425 mg/L), at 3, 6, 12 and 24 h in liver ($P = 0.009, 0.013, 0.034$ and 0.013 respectively, the concentrations were 0.6472685 ± 0.5256 mg/L vs 0.1554 ± 0.1043 mg/L, 0.8606826 ± 0.7155 mg/L vs 0.0014 ± 0.0029 mg/L, 0.0445 ± 0.0330 mg/L vs 0.0797 ± 0.1005 mg/L and 0.0863 ± 0.0399 mg/L vs 0.0034 ± 0.0075 mg/L respectively) and at 18 h in lung ($P = 0.009$, the concentrations were 0.0886 ± 0.0668 mg/L vs 0.0094 ± 0.0210 mg/L). There were no differences in 5-fluorouracil concentrations in renal tissue at each time-point.

CONCLUSION: The use of intraperitoneal 5-fluorouracil with HAES-Steri carrier solution provides a pharmacokinetic advantage for a local-regional killing of residual tumor cells and improve the accumulated penetrability of 5-fluorouracil with decreased systemic toxicity. Further clinical feasibility studies on the use of HAES-steri as carrier solution for intraperitoneal chemotherapy with 5-fluorouracil are warranted.

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Key words: Carrier solutions; Intraperitoneal chemotherapy; 5-fluorouracil; Pharmacokinetics; Tissue distribution

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INTRODUCTION

The gastrointestinal cancer tumor is one of the most common clinical malignant tumors^[1]. Although the surgical interventions have been advancing and radiotherapy, chemotherapy, biotherapy, immunotherapy and Chinese traditional medicine been developing all the time, the prognosis of patients with gastrointestinal cancer has not been improved obviously and the mortality not been decreased greatly so far, for which the main reason is that the regional recurrence and implantation metastasis can not be treated effectively. The recurrence and metastasis of gastrointestinal cancer often occurs in the resection place, peritoneal membrane surface and liver by turns^[2,3]. Intraperitoneal chemotherapy, which aims at this biological behavior and makes the recurrence and metastasis places of gastrointestinal cancer exposed to anti-cancer drug directly for a long time, provides a new adjunctive therapy for intraperitoneal malignant tumors, especially for gastrointestinal cancer now^[4,5]. However, two pharmacokinetic problems appear to limit the effectiveness of intraperitoneal therapy: poor tumor penetration and no uniform intraperitoneal distribution by the drug-containing solution^[6]. Several factors contribute to the drug distribution but intraperitoneal fluid volume is a dominant factor^[7]. Rosenheim *et al* have demonstrated in monkeys that small volumes of fluid do not flow freely in the peritoneum, even with multiple position changes^[8]. Volumes large enough to cause moderate abdominal distention result in more uniform intraperitoneal distribution. The ideal carrier solutions for intraperitoneal chemotherapy should expose cancerous surfaces or residual tumor cells within the peritoneal cavity to high levels of cytotoxic agent for as long as possible and make the agents distribute in the abdominal cavity uniformly^[9].

Current techniques for intraperitoneal chemotherapy administration most often utilize isotonic micromolecule solutions such as physiological saline, however, the low molecular weight of this solution results in its rapid peritoneal absorption and cannot make the system toxicity from intraperitoneal chemotherapy under satisfactory control^[10]. A successful attempt has been made to prolong retention of intraperitoneal chemotherapy by using icodextrin^[11-13], which is an isomolar glucose polymer-based dialysate solution. Another isomolar glucose polymer solution with a potentially long intraperitoneal dwell time is 6% hydroxyethyl starch^[14,15].

Slow clearance would benefit the use of cell cycle-specific drugs whose apoptotic effects enhance penetration in solid tumor. Agents undergoing extensive hepatic metabolism such as 5-fluorouracil and doxorubicin possess the greatest regional advantage with intraperitoneal instillation^[16-18].

HAES-steri is a neotype hetastarch with middle molecule, which is commonly used for clinical volume expansion therapy. On the basis of its characteristic that it can stay in the blood vessel for a long time and the research about the solutions of the same kind, HAES-steri is promising to be an ideal carrier solution for intraperitoneal chemotherapy. The purpose of these animal experiments was to determine the pharmacokinetics and tissue concentrations of 5-fluorouracil after intraperitoneal perfusion with two isotonic carrier solutions: physiologic saline, a low molecular weight solution, and HAES-steri, a middle molecular weight solution.

MATERIALS AND METHODS

Animals

Male and female Sprague Dawley rats weighing between 200 and 300 g were obtained from a single breeding colony (Laboratory Animal center of Southern Medical University). Animals were individually housed and were allowed free access to food and water. These experiments were conducted after approval by Laboratory Animal Center of Southern Medical University. (the license No. is SY × K 2006-0074).

Surgical procedure

All rats were briefly anesthetized by inhalation of ether (ether, Guanghua Chemistry Co., Ltd. Guangdong, China). Using a 50 mL injection syringe, the cytotoxic agent plus the carrier solution was administered intraperitoneally. The volume of solution administered was 0.1 mL/g body weight. Rats were returned to their cages to recover and were allowed free access to food and water. Rats were anesthetized by inhalation of ether for one minute before the end of the dwell-time. Through a midline thoracotomy the peritoneal fluid was withdrawn completely and quantitated. The blood samples of portal vein and inferior caval vein were taken with a 5 mL injection syringe and 2 mL syringe respectively, and tissue samples were taken from the stomach, colon, liver, kidney and lung.

Experimental design

A total of 60 rats were randomized into two groups according to the carrier solution administered. 5-fluorouracil (Nantong Jinghua Pharmaceutical Co., Ltd., China) was administered in tamed iodine. The dose of drug used in this study was chosen at 100 mg/kg, which exceeds the intraperitoneal dosage used in humans and was meant to be above the analytic detection limit in fluid samples. Based on this dosage, 5-fluorouracil was administered at a concentration of 1000 µg/mL. Two isotonic carrier solutions (0.1 mL/g body weight) were used: physiologic saline (Shenzhen Pharmaceutical Co., Ltd, Guangzhou, China) and 6% hetastarch (Beijing Fresenius Kabi Pharmaceutical Co., Ltd.). Each group was further randomized according to the length of the dwell period of chemotherapy (1, 3, 6, 12, 18 or 24 h). At the end of the procedure the rats were killed. A midline thoracoabdominal incision was made and all peritoneal fluid removed. The volume of peritoneal fluid was recorded and a 0.5 mL

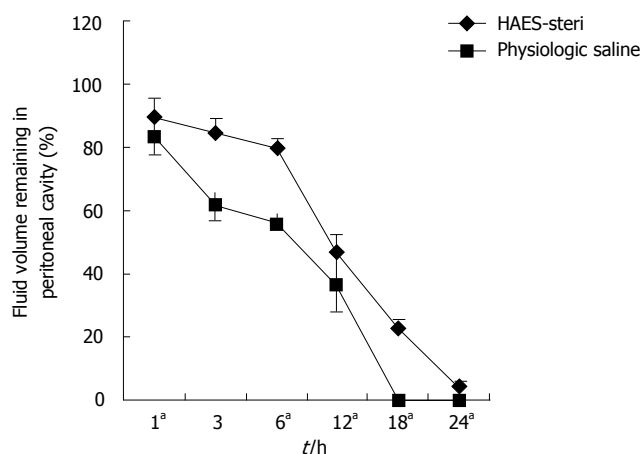


Figure 1 Mean peritoneal fluid volume remaining as a percentage of initial chemotherapy solution volume administered (^a $P < 0.05$ between the two carrier solutions).

sample was retained for analysis. Blood and tissue were also sampled. 5-fluorouracil concentrations in plasma, peritoneal fluid, and tissue samples were analyzed by high-performance liquid chromatography (HPLC).

HPLC analysis C

5-fluorouracil levels were determined in plasma, peritoneal fluid and tissue samples, using the HPLC procedures. The HPLC system consisted of a P200 high pressure constant flow pump, an UV-VIS detector set at 265 nm UV, along with an EC2000 color spectrum workstation, an EC2000 Chromatopac data processor. A reversed-phase Diamonsil C₁₈ 5 μ m silica column 250 mm \times 4.6 mm was used, coupled to a guard column of the same chemical consistency (Dikma Technologies, Beijing, China). The mobile phase consisted of a mixture of acetonitrile and ultrapure water (1:19, v/v), run at a flow rate of 1.0 mL/min. Sample injections were 20 μ L with 5-bromouracil as internal standard. All solvents used were HPLC grade (Merck, KGaA).

Sample preparation and analysis

Blood samples were centrifuged and the plasma was separated from the cells. Using a 15-mL polypropylene conical tube, a 500 μ L sample of plasma was treated with 100 μ L 5-bromouracil as internal standard and 2 mL acetoacetate (Guanghua Chemistry Co., Ltd., Guangdong, China) and mixed thoroughly in a vortex mixer. After centrifugation, the acetoacetate was transferred to another polypropylene tube and evaporated at approximately 40°C by blowing with a gentle stream of nitrogen. The residue was resuspended in 100 μ L mobile phase and filtered through a 0.45 μ m syringe filter before HPLC injection. Peritoneal fluid samples were treated as blood sample before HPLC injection.

Tissue samples were processed after drying surface moisture with filter paper. A sample of tissue was accurately weighed with electronic balance and homogenized in ultrapure water with the volume as 3 times as the weight of the sample (v:w = 3 mL/g) with a homogenizing machine. The tissue sample site was

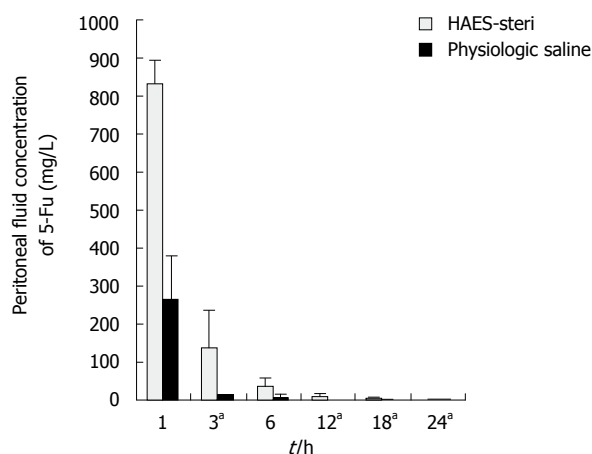


Figure 2 Mean peritoneal fluid concentration of 5-fluorouracil with different carrier solutions (^a $P < 0.05$ between the two carrier solutions).

consistent for all animals. The homogenate was centrifuged and the supernatant fluid was removed and the following was used as the blood sample.

Statistical analysis

The main parameters of pharmacokinetics and areas under the concentration-time curve were determined using DAS 2.0 (Drug and Statistics Software, Anhui, China). All pharmacokinetic data were compared between groups at each time-point with Mann-Whitney test (two-tailed) using SPSS 10.0. For all statistical procedures, P values < 0.05 were considered significant.

RESULTS

Intraperitoneal volume

Measurements of peritoneal fluid volume at each time-point showed slower clearance from the peritoneal cavity of HAES-steri when compared to physiologic saline (Figure 1). The mean percentage of fluid volume remaining in the peritoneal cavity was significantly higher with HAES-steri at 1, 6, 12, 18 and 24 h ($P = 0.047, 0.009, 0.005, 0.005$ and 0.005 respectively). No excess peritoneal fluid remained at 18 h with physiologic saline. At 24 h, the percentage of remaining peritoneal fluid volume with HAES-steri was 4.2%.

Peritoneal fluid drug concentration

At each time-point drug concentrations were determined within the peritoneal cavity (Figure 2). The mean peritoneal fluid 5-fluorouracil concentration was significantly greater at 3, 12, 18 and 24 h ($P = 0.009, 0.009, 0.005$ and 0.005 respectively). There was no significant difference in 5-fluorouracil concentrations of peritoneal fluid between carrier solutions at other time-points.

Plasma drug concentration in portal vein

Plasma 5-fluorouracil concentrations were significantly lower when the drug was administered with HAES-steri at 1 h ($P = 0.009$) and were significantly higher at 3, 12, 18 and 24 h ($P = 0.009, 0.034, 0.005$ and 0.019 respectively) (Figure 3A).

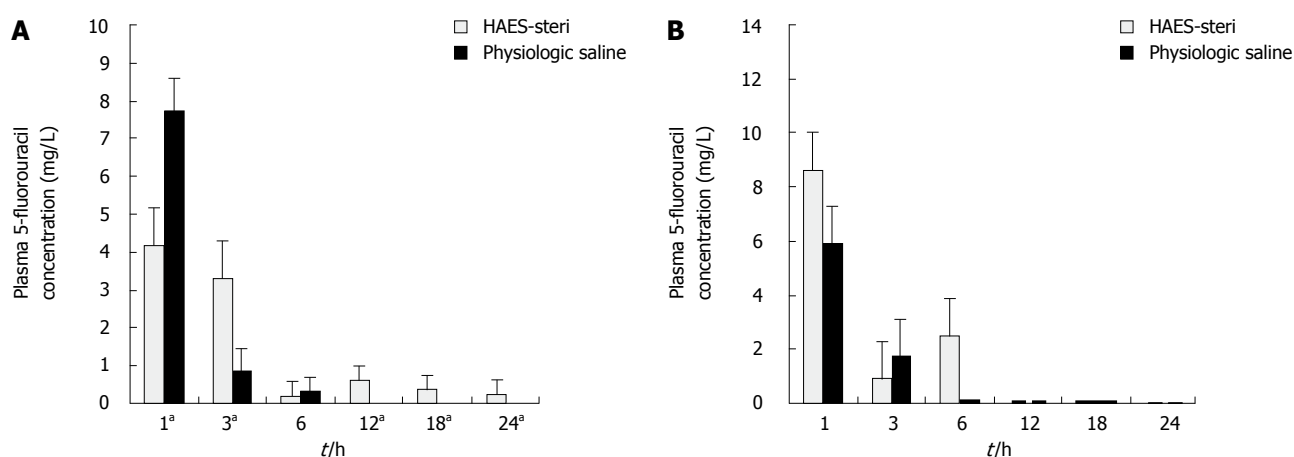


Figure 3 A: Mean plasma concentration of 5-fluorouracil in portal vein with different carrier solutions (^a $P < 0.05$ between the two carrier solutions); B: Mean plasma concentration of 5-fluorouracil in inferior caval vein with different carrier solutions.

Plasma drug concentration in inferior caval vein

There were no significant differences in plasma 5-fluorouracil concentration in inferior caval vein between different carrier solutions at each time-point (Figure 3B).

Total quantity of drug in peritoneal fluid

The mean total quantity of 5-fluorouracil in the peritoneal fluid decreased with time for both hetastarch and peritoneal dialysis solution, but was significantly greater with hetastarch at 12 h ($P = 0.008$), 18 h ($P = 0.009$) and 24 h ($P = 0.009$) (Figure 4A). No measurable drug or excess peritoneal fluid was present at 18 h when physiologic saline was used. When HAES-steri was used, the mean volume of peritoneal fluid remaining at 24 h was $4.2\% \pm 1.7\%$ (\pm SD) of the initial peritoneal fluid volume. The mean total quantity of 5-fluorouracil in this fluid was extremely low (0.7458 ± 0.1954 μ g).

Area under the curve ratio of peritoneal fluid to plasma 5-fluorouracil concentration

The area under the concentration over time curve (AUC) ratio with HAES-steri was 1551.095 for peritoneal fluid, 17.49 for plasma in portal vein and 19.466 for plasma in inferior caval vein. The AUC ratio for physiologic saline was 824.054 for peritoneal fluid, 14.516 for plasma in portal vein and 20.275 for plasma in inferior caval vein. The AUC ratio of peritoneal fluid to plasma in portal vein was 88.68 for HAES-steri, and 56.76 for physiologic saline. The AUC ratio of peritoneal fluid to plasma in inferior caval vein was 79.68 for HAES-steri, and 40.64 for physiologic saline. There was an increase of 156% in the AUC ratio of peritoneal fluid to plasma in portal vein and 196% in inferior caval vein with HAES-steri (88.68 *vs* 56.76 ; 79.68 *vs* 40.64).

Drug concentration in gastric tissue

Mean tissue concentrations of 5-fluorouracil were greater in gastric tissue with HAES-steri. These differences were significant at 18 h ($P = 0.016$). No significant differences were seen in gastric tissue concentrations of 5-fluorouracil at other time-points (Figure 4B).

Drug concentration in colon tissue

Tissue concentrations of 5-fluorouracil were significantly greater in colon tissue with HAES-steri at 18 h ($P = 0.009$) (Figure 4C). There were no significant differences in colon tissue concentrations of 5-fluorouracil at other time-points.

Drug concentration in liver tissue

Tissue concentrations of 5-fluorouracil were significantly greater in liver tissue with HAES-steri at 3, 6, 12 and 24 h in liver ($P = 0.009$, 0.013 , 0.034 and 0.013 respectively) (Figure 4D). There were no significant differences in liver tissue concentrations of 5-fluorouracil at other time-points.

Drug concentration in lung tissue

Tissue concentrations of 5-fluorouracil were significantly higher in lung tissue with HAES-steri at 18 h ($P = 0.009$) (Figure 4E). There were no significant differences in lung tissue concentrations of 5-fluorouracil at other time-points.

Drug concentration in renal tissue

No significant differences were seen in renal tissue concentrations of 5-fluorouracil with the two carrier solutions at each time-point (Figure 4F).

DISCUSSION

Intraperitoneal chemotherapy has shown certain benefits as a treatment for peritoneal surface malignancies and regional recurrence of gastrointestinal cancer after operation so far^[19-21]. However, no standard treatment in terms of schedule, dwell-time, drug or carrier solution has been established^[9]. It remains an unrealized goal that intraperitoneal chemotherapy makes a great stride in improving the survival and prognosis of patients with gastrointestinal cancer. The lack of ideal carrier solutions is one of the main problems which prevent the progress of intraperitoneal chemotherapy. After radical or palliative surgery for gastrointestinal cancer, tumor recurrence within the peritoneal cavity may be due to residual tumor nodules on the peritoneal surface or to implantation of free cancer cells circulating within peritoneal fluid.

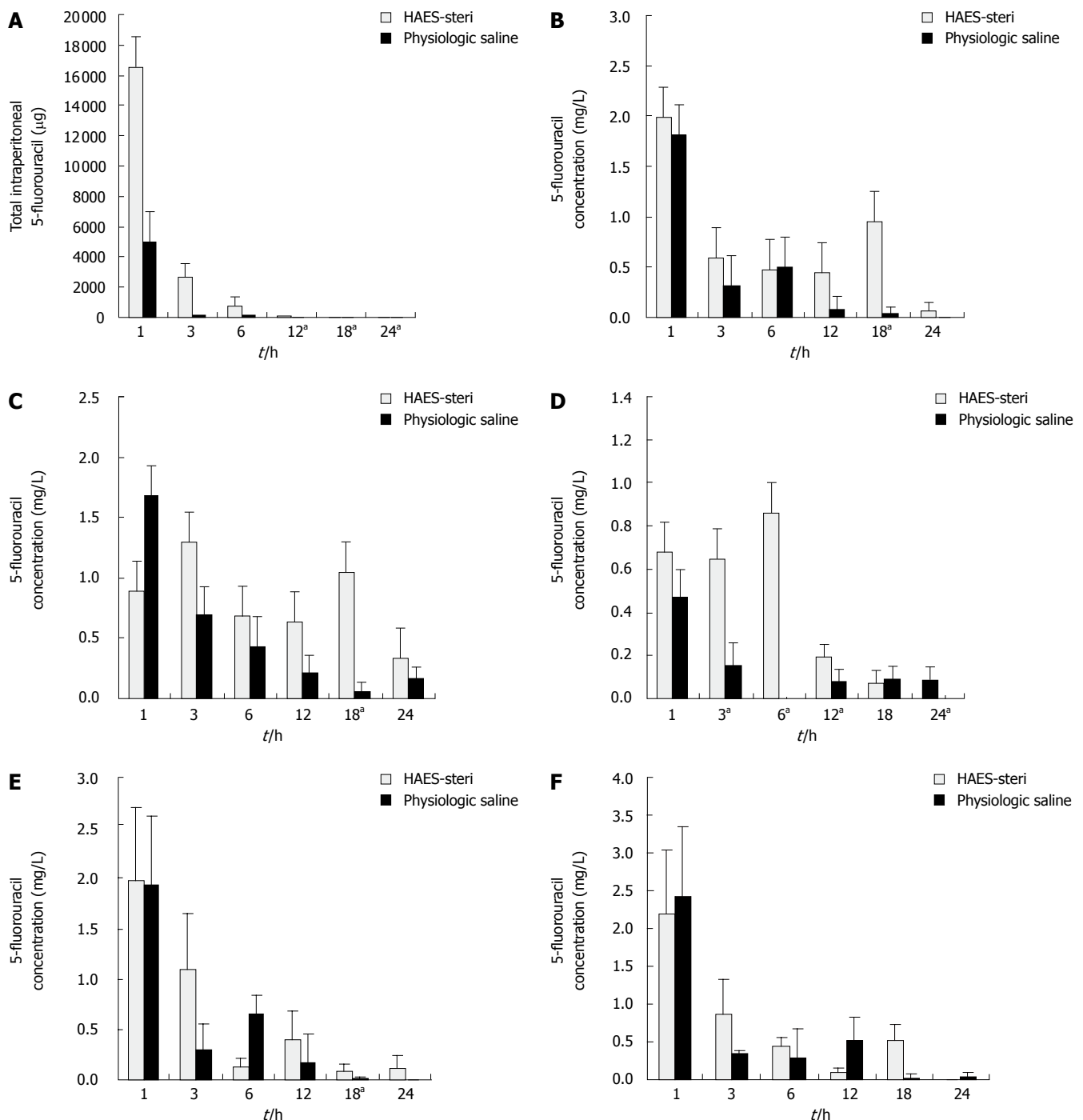


Figure 4 A: Mean total quantity of 5-fluorouracil in peritoneal fluid with different carrier solutions ($^aP < 0.05$ between the two carrier solutions); B: 5-fluorouracil concentration in gastric tissue with different carrier solutions ($^aP < 0.05$ between the two carrier solutions); C: 5-fluorouracil concentration in colon tissue with different carrier solutions ($^aP < 0.05$ between the two carrier solutions); D: 5-fluorouracil concentration in liver tissue with different carrier solutions ($^aP < 0.05$ between the two carrier solutions); E: 5-fluorouracil concentration in lung tissue with different carrier solutions ($^aP < 0.05$ between the two carrier solutions); F: 5-fluorouracil concentration in renal tissue with different carrier solutions ($^aP < 0.05$ between the two carrier solutions).

Preventing recurrence effectively requires that the tumor nodules have prolonged exposure to the cytotoxic drug. It is necessary for chemotherapy solutions to distribute evenly throughout the entire peritoneal cavity for a prolonged period in order to treat peritoneal and visceral surfaces safely and successfully.

However, isotonic salt solutions, the traditional carrier solutions in use for intraperitoneal chemotherapy, tend to be rapidly absorbed due to their low molecular weight. Pestieau and colleagues have proved that isotonic 0.9%

sodium chloride was cleared more rapidly from the peritoneal cavity than high molecular weight solutions and hypertonic sodium chloride, when used as carrier solutions for intraperitoneal chemotherapy with 5-fluorouracil and gemcitabine in an animal model^[9]. The inability of isotonic salt or dextrose solutions to maintain a prolonged high intraperitoneal fluid volume limits their effectiveness as carrier solutions for intraperitoneal chemotherapy. The osmolality of the solution may play a role in prolonging the dwell time of intraperitoneal chemotherapy. In a

study by Litterst *et al*, it was shown that slightly hypertonic carrier solutions can prolong the peritoneal retention of chemotherapeutic agents within the peritoneal cavity, probably by inducing a fluid shift inward to the peritoneal cavity^[22]. Although the increased accumulation of drugs in tumor cells and enhanced cytotoxicity of cisplatin in hypotonic solution have been confirmed *in vitro* and *in vivo*^[23,24], the clinical success with hypotonic solutions as carrier solutions for intraperitoneal chemotherapy has been limited^[25,26]. It has been shown that high molecular weight carrier solutions such as Icodextrin and hetastarch have the ability to maintain high intraperitoneal volume for a longer period. Preliminary data receiving intraperitoneal chemotherapy with 7.5% Icodextrin showed that a similar quantity of fluid was drained from the peritoneal cavity as was originally instilled 24 h after administration^[27]. However, net fluid flow into the peritoneal cavity may occur when 7.5% icodextrin solutions were used, which could decrease the concentration of drug exposed to cancerous surfaces^[6]. A clinical study on the fluid dynamics of 4% icodextrin as carrier solution for intraperitoneal chemotherapy showed that it maintained its instilled volume for up to 48 h, and half the instilled volume remained after 72 and 96 h^[28]. Another high molecular weight carrier solution, 6% hetastarch, has also been used in a recent clinical study, which showed reduced clearance of hetastarch from the peritoneal cavity when compared with 1.5% dextrose peritoneal dialysis solution^[29].

In the study reported here, the use of HAES-steri, a starch-based carrier solution with middle molecular weight, reduced the clearance of chemotherapy solution from the peritoneal cavity when compared to physiologic saline (0.9% sodium chloride solution). The mean percentage of fluid volume remaining in the peritoneal cavity was significantly higher with HAES-steri at 1, 6, 12, 18 and 24 h. The total quantity of intraperitoneal drug at each time-point was also higher with HAES-steri, especially at 12 h and 18 h. By delaying the clearance of intraperitoneal fluid and thereby maintaining a large distribution, HAES-steri may maximize exposure of cancerous surfaces and optimize intraperitoneal chemotherapy treatments. At the 3, 12 and 18 h time-point, a significantly increased concentration of intraperitoneal 5-fluorouracil was demonstrated. It indicated that HAES-steri could reduce the clearance of 5-fluorouracil from peritoneal cavity and increase the drug concentration exposed to peritoneal surfaces at the same time as this solution maintained high volume in peritoneal cavity for a long time. Accordingly, a larger number of residual tumor cells, minute nodules or free cancer cells in peritoneal cavity can be attacked by high concentrations of anti-cancer drug for over a given time period, which may improve the effectiveness of intraperitoneal chemotherapy for peritoneal regional recurrence of gastrointestinal cancer after surgery.

An important parameter for pharmacokinetic analyses of a drug is the AUC, which represents the total drug exposure integrated over time^[10]. The AUC is traditionally the relationship between time and plasma concentration, but can also be applied to concentration of drug in peritoneal fluid for intraperitoneal chemotherapy. Cancer chemotherapy pharmacokinetics assumes a definite

relationship of drug response to drug dose. Following intraperitoneal administration of a drug, the AUC reflects the degree of exposure of peritoneal surfaces to chemotherapeutic agent. It is the best estimate of drug delivery and a predictor of response. By comparing the AUC of a drug after intraperitoneal administration in HAES-steri to the AUC after administration in physiologic saline, an estimate of the optimum carrier solution that will prolong contact of peritoneal surfaces and residual tumor cells with chemotherapy solution can be obtained. The ratio of the AUC of 5-fluorouracil in peritoneal fluid to that in plasma of inferior caval vein after intraperitoneal administration reflects exposure of peritoneal surfaces to chemotherapy solution in relation to plasma concentrations of drug, which influences systemic toxicity. The higher AUC ratio of peritoneal fluid to plasma 5-fluorouracil concentration with HAES-steri suggests that better regional exposure of 5-fluorouracil and lower systemic toxicity can be achieved than with physiologic saline. Using intraperitoneal 5-fluorouracil with HAES-steri provides a potential for a favorable antitumor effect on small peritoneal surface tumor deposits or microscopic residual disease.

Another advantage of intraperitoneal chemotherapy is to prevent liver metastasis of gastrointestinal cancer after operation with chemotherapeutics of high concentration in portal vein and liver. In this study, although plasma 5-fluorouracil concentrations were significantly lower when the drug was administered with HAES-steri than with physiologic saline at 1 h, those were significantly higher at 3, 12, 18 and 24 h. Moreover, concentrations of 5-fluorouracil were significantly greater in liver tissue with HAES-steri at 3, 6, 12 and 24 h. This shows that HAES-steri has advantages over physiologic saline as carrier solutions for intraperitoneal chemotherapy to kill free cancer cells in portal vein, consequently, using HAES-steri as carrier solution for intraperitoneal chemotherapy with 5-fluorouracil may improve the effectiveness to prevent liver metastasis of gastrointestinal cancer after surgery.

Tissue concentrations of 5-fluorouracil were significantly higher in gastric and colon tissue at 18-h time-point when HAES-steri was used as carrier solution. This would suggest that HAES-steri increases the accumulated penetrated activity of 5-fluorouracil when used as carrier solution for intraperitoneal chemotherapy, which may benefit the eliminating internal cancer cells in residual tumor nodules. No significant differences were seen in renal tissue concentrations of 5-fluorouracil with the two carrier solutions at each time-point and there were no significant differences in lung tissue concentrations of 5-fluorouracil at time-points except 3-h. These may indicate again that HAES-steri makes the systemic toxicity of intraperitoneal chemotherapy under control when used as carrier solution.

Another advantage of HAES-steri as carrier solution for intraperitoneal chemotherapy is that maintenance of an expanded intraperitoneal space with the use of HAES-steri may additionally ensure separation of loops of bowel to allow direct contact of chemotherapy solution with bowel surfaces prone to adhesion formation and subsequent disease recurrence^[30]. The reduction in adhesion formation

has been shown with the use of intraperitoneal 4.5% icodextrin lavage and instillation after laparoscopic gynecologic surgery^[18]. The further pathologic study of the impact on the peritoneum and the healing of the operative incision when HAES-steri is used intraperitoneally is needed.

In brief, this study suggests that HAES-steri, by remaining longer in the peritoneal cavity, provides wider intraperitoneal distribution of 5-fluorouracil, and an increased exposure of peritoneal surfaces to anti-cancer drug with lower systemic toxicity than physiologic saline. HAES-steri is a promising carrier solution for intraperitoneal chemotherapy and further clinical feasibility studies on the use of HAES-steri as carrier solution for intraperitoneal chemotherapy with 5-fluorouracil are warranted.

COMMENTS

Background

The principal pharmacokinetic advantage of intraperitoneal chemotherapy over intravenous chemotherapy is the high local drug concentration with low systemic toxicity. The traditional carrier solutions for intraperitoneal chemotherapy fail to optimize this advantage, because these are prone to be cleared from peritoneal cavity rapidly. The ideal carrier solutions should expose cancerous surfaces or residual tumor cells within the peritoneal cavity to high levels of the cytotoxic agent as long as possible.

Research frontiers

It has been shown in published articles that high molecular weight solutions have the ability to maintain high intraperitoneal volume for a longer period, which may offer a number of advantages over low molecular weight solutions. Further study on the pharmacokinetics of intraperitoneal chemotherapy with middle or high molecular weight solutions may solve the problem that there are no ideal carrier solutions for intraperitoneal chemotherapy.

Innovations and breakthroughs

In this article, we studied and compared the pharmacokinetics and tissue distribution of intraperitoneal 5-fluorouracil with HEAS-steri, a neotype 6% hydroxyethyl starch as carrier solution, and physiologic saline (0.9% sodium chloride solution), a traditional carrier solution, in rats, which may provide a promising carrier solution for intraperitoneal chemotherapy.

Applications

This study on the pharmacokinetics and tissue distribution of intraperitoneal 5-fluorouracil with HEAS-steri and physiologic saline offers experimental data for further clinical study and application of HEAS-steri as carrier solution for intraperitoneal chemotherapy.

Terminology

Intraperitoneal chemotherapy, a treatment in which anticancer drugs are put directly into the abdominal cavity through a thin tube, has obvious pharmacokinetic advantages over intravenous chemotherapy when used in palliative therapy for peritoneal carcinomatosis from gastrointestinal carcinoma and prevention of the postoperative peritoneal recurrences and metastases of gastrointestinal cancer.

Peer review

In the present study the effect HAES-steri, as a promising carrier solution for intraperitoneal chemotherapy, was investigated. The study showed that HAES-steri delayed the clearance of intraperitoneal fluid and increased the concentration of 5-fluorouracil in the peritoneal fluid, tissue (especially liver) and portal vein. The manuscript deals with an interesting topic and conclusive results.

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Prospective cohort comparison of flavonoid treatment in patients with resected colorectal cancer to prevent recurrence

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Abstract

AIM: To investigate biological prevention with flavonoids the recurrence risk of neoplasia was studied in patients with resected colorectal cancer and after adenoma polypectomy.

METHODS: Eighty-seven patients, 36 patients with resected colon cancer and 51 patients after polypectomy, were divided into 2 groups: one group was treated with a flavonoid mixture (daily standard dose 20 mg apigenin and 20 mg epigallocatechin-gallat, $n = 31$) and compared with a matched control group ($n = 56$). Both groups were observed for 3-4 years by surveillance colonoscopy and by questionnaire.

RESULTS: Of 87 patients enrolled in this study, 36 had resected colon cancer and 29 of these patients had surveillance colonoscopy. Among the flavonoid-treated patients with resected colon cancer ($n = 14$), there was no cancer recurrence and one adenoma developed. In contrast the cancer recurrence rate of the 15 matched untreated controls was 20% (3 of 15) and adenomas evolved in 4 of those patients (27%). The combined recurrence rate for neoplasia was 7% (1 of 14) in the treated patients and 47% (7 of 15) in the controls ($P = 0.027$).

CONCLUSION: Sustained long-term treatment with a flavonoid mixture could reduce the recurrence rate of colon neoplasia in patients with resected colon cancer.

INTRODUCTION

Patients with resected colon cancer are at risk of cancer recurrence which depends mainly on the tumor stage^[1]. Within 4-5 years after a curative surgical resection about 40%-50% of patients suffer from a tumor recurrence when their initial tumor stage was II or III according to the International Union against Cancer (UICC) classification^[2-4]. Tumor recurrence can manifest itself as a local recurrence at the site of resection, as metachronous tumor growth somewhere else in the colon or as local or distant metastasis. Recurrence in the colon can take three forms of neoplasia: either as incident carcinoma, as incident adenoma or as a mixture of both.

Patients with colon polyps (adenomas, hyperplastic polyps or serrated polyps) who had a polypectomy are also at risk of recurrence^[5]. After an index polypectomy these patients can develop incident adenomas in 40% of cases within 3 years depending on the histology of the polyp. The adenoma recurrence is highest for large and multiple adenomas with dysplastic changes of the adenoma structure^[5].

There is much controversy about what can be done to reduce the risk or recurrence of neoplasia in tumor and polyp patients. Secondary prevention is urgently needed in these patients; however, it is not yet clear what measures are most effective. Epidemiological studies indicate that dietary interventions with ballast augmented food can be successful for primary prevention of colorectal carcinomas^[6]. On the other hand diets supplemented with bran^[7] and fruits and vegetables^[8] do not suppress the

evolution of colorectal adenomas after polypectomy. Other dietary components such as folic acid, calcium, vitamin D and selenium either have shown only marginal beneficial effects or no effects for prevention^[9-12]. Antioxidative vitamins could not prevent gastrointestinal cancer^[13]. Beside dietary factors (bioprevention) chemically defined intervention with aspirin^[14-17] and nonsteroidal anti-inflammatory drugs (NSAIDs) seem to be effective for primary and secondary prevention of colon neoplasia^[18,19]. However, their unwanted side effects and complications (ulcerations, bleedings and thromboembolic events) prevent their general use for risk reduction^[20].

Various modes of bioprevention with dietary components have been tested mostly in epidemiological studies^[21-23] and in studies with cell culture work and other *in vitro* tests^[24]. Few clinical intervention studies were reported. Candidates for use in clinical studies include secondary plant products such as flavonoids, indols, isothiocyanates, glucosinolates, allyes, resveratrol, curcumin, saponins and terpenes. Some of these plant products can be applied as nutritional supplements as tablets, thereby facilitating long-term use without side effects or problems of compliance. Tea flavonoids from green tea and camomile contain flavons, flavonols and flavanols. They have been shown to display various anticarcinogenic, antiproliferative, antimutagenic and antioxidative properties *in vivo* and *in vitro*^[24]. Certain species of dietary flavonoids were able to reduce the risk of colorectal cancer^[25], even in a dose dependent manner^[26]. This is not true for colon neoplasia in general^[27,28]. Recently, a clinical study^[29] suggested, that the flavonol quercetin taken together with curcumin suppressed the growth of adenomatous polyps in patients with hereditary colon polyposis syndrome (familial adenomatous polyposis, FAP patients).

We decided to study prospectively the effects of a sustained treatment with a tea-based flavonoid mixture on the evolution of neoplastic alteration in a cohort of high risk patients with resected colon cancer as well as patients after polypectomy. In this proof of principle study we found that a flavonoid intervention can reduce the recurrence rate of neoplasia in patients with sporadic colorectal neoplasia, in comparison with an untreated matched control group.

MATERIALS AND METHODS

Study subjects

Between January 2000 and December 2003 a total of 160 patients with colorectal neoplasia (index patients) were recruited and their data were collected from the clinical charts of the Community Hospital Groß-Gerau, Germany (Department of Internal Medicine and General Surgery) and included into the tumor registry for this clinical study. All patients with the diagnosis of colorectal cancer and colon polyps confirmed by pathology reports were eligible for this study if they completed the clinical questionnaire and had surgical tumor resection or polypectomy. By the end of 2003, recruitment was terminated and until December 2005, 87 patients were followed up by surveillance colonoscopy. Patients who were still in the

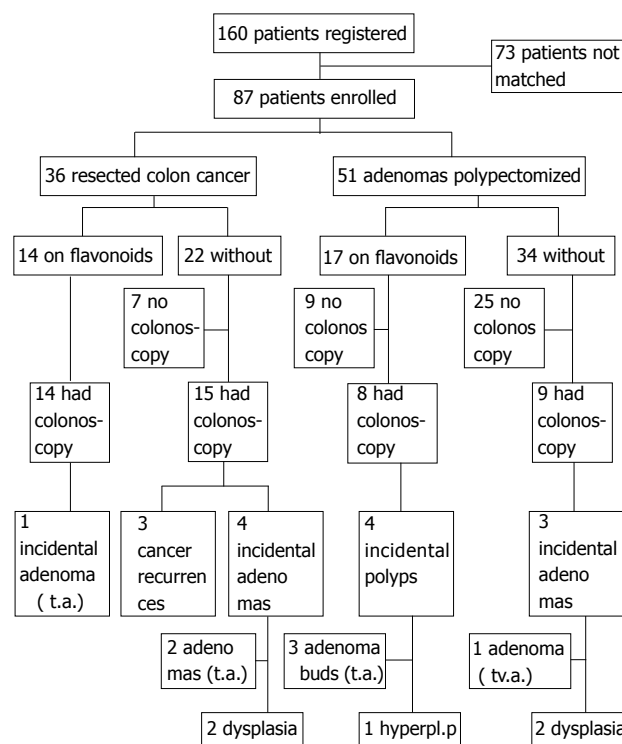


Figure 1 Trial profile and outcomes. t.a.: Tubular adenoma; tv.a.: Tubulovillous adenoma; hyperpl.p: Hyperplastic polyp, buds diminutive polyp (< 5 mm).

study at this time were considered to be censored cases for overall survival. All data were extracted from the local tumor registry of the clinic and further pseudonymized for evaluation. In this prospective observational cohort study the investigators had no role in the clinical management; all treatment decisions (except the assignment of the flavonoid nutritional supplementation) and the schedule of surveillance colonoscopies were left at the discretion of the treating physician. Surveillance data were collected prospectively. All 160 patients were treated according to the clinical guidelines for follow-up investigations for colorectal cancer and colon adenomas published by the German Association for Digestive and Metabolic Diseases^[30]. This study was approved by the Ethics Committee of the Technical University of Dresden, Germany. The patients provided information using a self-administered questionnaire and in this way written informed consent was obtained authorizing use of their data for this study.

Study protocol

Figure 1 explains the trial profile, the outcome and the patient flow of this controlled study. One hundred and sixty patients were registered and of these 87 patients were enrolled to test the efficacy of flavonoids. During the four years from January 2000 to December 2003 we recruited 31 of the 160 patients to agree to take the flavonoid supplement for tumor prevention. Following assignment to treatment these 31 patients were matched to 56 controls. Matching by gender, age (10 years intervals) and type of neoplasia (resected carcinoma *vs* polypectomized adenomas) was performed by using the data of the 129 untreated patients. The remaining 73 patients of the

Table 1 Demographic and outcome data of patients treated with flavonoids and controls

	Treated (<i>n</i> = 31)	Controls (<i>n</i> = 56)	<i>P</i> value
Males/females	17/14	31/25	> 0.9
Age (yr) median (IQR)	74 (68-80)	77 (69-82)	0.35
BMI (kg/m ²) median (IQR)	26.1 (24.4-28.2) (<i>n</i> = 28)	27.5 (25.0-30.3) (<i>n</i> = 45)	0.32
Resected colon cancer/polypectomy	14/17	22/34	0.65
Surveillance colonoscopy/no	22/9	24/32	0.014 ¹
Years: Median (IQR)	3.5 (3-4.75) (<i>n</i> = 22)	3.0 (2-3) (<i>n</i> = 24)	0.019 ¹
Surveillance time by questionnaire			
Years: Median (IQR)	3.6 (3.1-4.7)	2.9 (2.5-3.4)	0.004 ¹
Cancer recurrence/no	0/20	3/18	0.23
Polyp recurrence/no	5/15	7/14	0.73
Neoplasia recurrence/no	5/15	10/11	0.20
Smoker/non-smoker	2/27	6/48	0.71
Alcohol/no	24/5	33/20	0.08
Black tea/no	16/15	27/26	> 0.9
Green tea/no	13/16	21/27	> 0.9
Fruit intake < 3/≥ 3 × weekly	8/20	17/35	0.80
Vegetable intake < 3/≥ 3 × weekly	15/13	29/22	0.82
Aspirin use/no	11/20	18/37	0.82
NSAID use/no	2/29	3/52	> 0.9
Colon cancer in family/no	1/30	6/49	0.41
Adenomas in family/no	2/29	1/54	0.29

IQR: Interquartile range (25%-75%); BMI: Body mass index; *n*: Number of patients; ¹Significantly different at *P* < 0.05.

total of 160, who did not fulfil the matching criteria, were not followed further in this study. The flavonoid-treated patients took a daily dose of 2 tablets of the flavonoid mixture^[24] containing 10 mg apigenin and 10 mg epigallocatechin-gallate per tablet. This nutritional supplement (tea bioflavonoids) was produced according to the principles of Good Manufacturing Practice by Köhler-Pharma, Alsbach-Hähnlein, Germany. The content of active ingredients in each batch of the product was tested by chemical analysis (HPLC technique). Flavonoids were taken for 2-5 years; the treatment compliance was evaluated by questionnaire.

Outcomes were evaluated according to the per protocol principle: data of patients using flavonoids (*n* = 31) were analyzed regardless of how long they had been treated. The primary endpoints of this study were the incident neoplasia (cancer and/or adenomas) observed by surveillance colonoscopy.

The self-administered questionnaire provided information on relevant clinical variables which might influence the clinical outcome. These included life style variables, body mass index (BMI), a dietary food frequency questionnaire, information on medical treatment, cancer and adenoma histories of relatives and tea consumption (Table 1). Data on colonoscopy findings were taken from the standardized clinical endoscopy protocols and transferred to the registry. Histological findings of neoplasia provided by the clinical pathologist were rated according to the guidelines as mentioned above^[30]. Tumor stage was assessed from the surgical protocols and rated according to the UICC classification^[4].

Statistical analysis

The data of the total cohort of 160 patients were subdivided into the two basic sub-cohorts: patients only observed (*n* = 73) and patients surveyed for secondary

prevention (*n* = 87). The latter group was divided into a treatment group (*n* = 31) and a control group (*n* = 56) as described in the Study Protocol. The patient characteristics of the two surveillance groups, the per protocol group of the treated patients (*n* = 31) and their controls (*n* = 56) were compared on baseline as well as for their outcome variables by using descriptive and confirmatory statistical methods. Categorical variables were analyzed using the chi-square test or the 2-sided Fisher Exact Test in the case of small frequencies. Continuous variables (age, BMI) were analyzed using the non-parametric Wilcoxon-Mann-Whitney *U*-Test. They are described by their median and the interquartile range (IQR). The IQR is defined as the range between the 25th and the 75th percentile of the empirical distribution of the data.

Differences of recurrence were expressed in percentages as absolute differences. The relative risk ratio (RRR) and the number needed to treat (NNT) were computed. Because of the observational nature of this study no adjustments for multiplicity were applied and *P* < 0.05 was considered statistically significant.

RESULTS

The prognostically relevant clinical variables of the treated patients were compared with those of the matched patients (Table 1). During the study period one patient in the treatment group and two patients in the control group died of causes not related to tumor recurrence. The patients in the treated group had significantly higher numbers of follow-up colonoscopies than patients in the control group (Table 1). The time under surveillance both by colonoscopy and by questionnaire was significantly longer for the treatment group (Table 1). The ratio of cancer to polyp patients was not significantly different (45% *vs* 39%) among treatment and control group.

Table 2 Comparison of clinical variables in patients with resected colon cancer on surveillance colonoscopy treated with flavonoids *vs* controls

	Flavonoid treatment (<i>n</i> = 14, %)	Controls (<i>n</i> = 15, %)	<i>P</i> value
Males/females	7/7	7/8	> 0.9
Age (yr) median (IQR)	75.0 (77-82)	81.0 (77-86)	0.12
BMI (kg/m ²) median (IQR)	26.2 (24.6-28.0) (<i>n</i> = 13)	25.9 (24.5-27.5) (<i>n</i> = 10)	0.57
Smoker/non-smoker	0/13 (0)	1/12 (8)	> 0.9
Alcohol habitual/no	13/0 (100)	7/5 (58)	0.015 ¹
Black tea/no	5/9 (36)	8/5 (61)	0.26
Green tea/no	5/8 (36)	5/6 (45)	> 0.9
Fruit intake < 3/≥ 3 d a week)	2/11 (15)	2/10 (17)	> 0.9
Vegetable intake < 3/≥ 3 d a week)	6/7 (46)	8/4 (67)	0.43
Aspirin/no	4/10 (28)	7/7	0.44
NSAID/no	0/14 (0)	1/13 (7)	> 0.9
Colon <i>vs</i> rectum cancer	13/1 (93)	9/6 (60)	0.080
Low <i>vs</i> high tumor stage (I and II / III)	9/5 (64)	9/6 (60)	> 0.9
Surveillance time by colonoscopy			
Years: median (IQR)	4.0 (3.25-5)	3.0 (2-3)	0.022 ¹

IQR: Interquartile range (25%-75%). ¹Significantly different at *P* < 0.05.

Table 3 Recurrence rates of colon neoplasia in patients with resected colon cancer treated with flavonoids compared to controls

	Treated (% of total, <i>n</i> = 14)	Controls (% of total, <i>n</i> = 15)	Absolute difference (%)	RRR	NNT	<i>P</i> value
Cancer recurrence/no	0/14 (0)	3/12 (20)	20		5	0.125
Adenoma recurrence/no	1/13 (7)	4/11 (27)	20	3.9	5	0.101
Neoplasia recurrence/no	1/13 (7)	7/8 (47)	40	6.7	2.5	0.027 ¹

RRR: Relative risk ratio; NNT: Number needed to treat. ¹Significantly different at *P* < 0.05.

Recurrence rates of cancer were 0 in 20 in the treated group *vs* 3 in 21 in the control group (*P* = 0.23). Polyp recurrence rates were 5 in 20 in the treatment group *vs* 7 in 21 in the control group (*P* = 0.73). The combined rate of recurrence for neoplasia was 5 in 20 in treated *vs* 10 in 21 in the controls (*P* = 0.20). These differences are not statistically significant, but there is a trend for more favourable outcomes in the flavonoid exposed patients. Note that both groups were not adjusted according to surveillance colonoscopy and according to neoplasia type. The sample size of this proof of principle study is small. Also, it can be seen in Figure 1, that the incident polyps in the control group were high grade adenomas (4 adenomas with dysplasia, one tubulovillous adenoma); there were only 2 tubular adenomas. Among the treated patients there were 3 diminutive tubular adenomas (polyp buds), one hyperplastic polyp and one tubular adenoma (with 10 mm diameter). This shows that there were more advanced adenomas present in the control group than in the treatment group.

Fruit consumption of less than 3 d a week was considered as low intake and was found in 29% (8 in 28) of the treatment group as compared to 33% (17 in 52) of the control group (*P* = 0.80). Habitual vegetable intake of less than 3 d a week was reported by 54% (15 in 28) of patients in the treatment group *vs* 57% (29 in 51) in the control group. Habitual drinking of green and black tea was not significantly different among both groups; about 44% drank green tea and 51% black tea. About 10% of the patients in both groups smoked and about 30% of them took aspirin regularly. NSAIDs were taken long

term by 5%-6% of the patients in both groups. Habitual alcohol use was reported by 83% in the treatment group as compared to 62% in the control group (*P* = 0.08). Gender, age and BMI were approximately evenly distributed among the two groups.

Most patients in the flavonoid group (20 in 31) took the nutritional supplement for more than 12 mo, 8 patients took it less than 3 mo, 2 up to 6 mo and one patient up to 12 mo. Three in 27 (11%) reported slight discomfort and discontinued the flavonoid treatment within 3 mo. The majority of 65% (17 in 26) took the flavonoids continuously on a daily basis.

As the data in Table 1 suggested that there is a possible treatment effect of the use of flavonoids we analyzed our data in the well adjusted group of patients with curative colon cancer resection. There were 14 patients with resected colon cancer in the treatment group compared to 15 control patients (Table 2); all had surveillance colonoscopies. None of the treated patients had cancer recurrence *vs* 20% (3 in 15) of the controls. Among the controls two patients had metastatic colorectal cancer and one had local cancer recurrence at the surgical anastomosis. The time to relapse was 2-3 years after surgery in patients with cancer recurrence. Adenomas developed in 7% (1 in 14) of the treated patients and in 27% (4 in 15) of the controls including two adenomas with dysplasia (Table 3). There was a statistically significant difference (*P* = 0.02) between the two groups when the combined endpoint of neoplasia recurrence (incident cancer and incident adenomas) was evaluated. The potentially confounding patient characteristics of both groups did not differ

significantly except for habitual alcohol consumption, which was significantly more prevalent in the treated patients than in controls. For neoplasia recurrence the prognostically most important factor is the previous tumor stage, which was not significantly different between the two groups.

DISCUSSION

Recurrence risk is the main concern of patients with previous resected colorectal cancer^[1-4]. On follow-up about 40% of surgically curable colorectal cancers with stage II and stage III (according to the UICC staging system) will suffer recurrent cancers within 3-4 years. The best outcomes were reported for stage I and stage II tumors (around 90% survival without recurrences). The prognosis of stage III cancer (with cancerous regional lymph nodes) is less favourable, but can be improved by adjuvant chemotherapy. Treated cases and controls in our study did not differ regarding the initial tumor stage at surgery; about 40% in both groups were stage III tumors, only 2 of them (controls) had adjuvant chemotherapy because the surgeon felt confident that most of these patients would not be suitable for adjuvant chemotherapy. The tumor recurrence in the controls was not observed in the patients on chemotherapy, but there were too few patients to judge whether this could influence outcomes. We found the expected recurrence rate in the controls (Table 3), but no incident cancers and only one incident adenoma in the flavonoid exposed patients. Eighty-seven of the 160 patients from the registry were enrolled because we detected only 56 controls that could be properly adjusted to the 31 treated patients. The matching ratio of about one to two (31 treated *vs* 56 controls) seems to be appropriate. In this real world study 76% of the treated and 43% of the controls had surveillance colonoscopies; among the resected patients 80% had surveillance by colonoscopy but only 33% of the polypectomized patients. This fact might influence the reliability of the conclusions regarding the adenoma recurrence.

Our controlled clinical trial was a prospective and observational cohort study performed with the aim of finding out whether long-term flavonoid exposure of patients from a tumor registry alters the outcome compared to untreated control patients. This proof of principle study suggests that flavonoids can be used to reduce the recurrence rate in patients with resected colorectal cancers. Flavonoids are good candidates for primary and secondary prevention of colorectal cancer, since numerous *in vitro* studies and animal work report on their beneficial activities in terms of suppression of cancer proliferation, antioxidative and antiangiogenic properties^[24]. Epidemiological investigations^[22,25,26], *in vivo* and *in vitro* experiments^[31-35] and one clinical intervention study^[29] support this concept. Other authors could not find protective effects of flavonoids on colorectal cancer incidence^[21,27,36]. Flavonoids derived from tea plants can be used as a mean of bioprevention and have been manufactured and marketed as nutritional supplements^[24]. Other methods of prevention are not effective (e.g. vitamins except folic acid), show only marginal efficacy (e.g. calcium, selenium) or cannot be used in general because of their unwanted side effects and complications

(aspirin, NSAIDs)^[20].

We tested the efficacy of flavonoid supplementation in a high risk population (resected colorectal cancer) to examine its effect in a relatively small number of patients, which were carefully adjusted for various clinical variables with prognostic relevance. However, there are prognostic clinical factors which were not taken into account such as penetration depth into the colonic wall and histological grading. Clinical studies with a larger sample size and a higher statistical power are necessary to show that flavonoid exposure alters the outcome in terms of tumor recurrence. Flavonoids could prevent recurrences of neoplasia by protecting the genome of colonocytes from genotoxic insults such as oxidative damage, free radical attacks and adduct formation^[37]. Flavonoids are secondary plant products which could be responsible for some of the healthy effects of fruits and vegetables. It is still unknown which components of vegetables and fruits are effective for tumor prevention; ballast, fibres and secondary plant products play a major role^[6,10,38]. Flavonoids, indols, isothiocyanates, curcumin, resveratrol, glucosinolates and other plant products affect carcinogenic, mutagenic and neoplastic mechanisms^[24], but could also induce protective enzymes of the intestinal mucosa^[39]. Beside the type of chemical and biological prevention lifestyle factors, type and amount of tea consumption, genetic factors, aspirin and NSAID medication could influence the outcome. These variables have to be considered when evaluating the effects of flavonoid intervention. As shown in Tables 1 and 2 these variables were well balanced among cases and controls. However, alcohol use was more prevalent in the treated patients with resected colorectal cancer than in controls. We do not think that differences of habitual alcohol drinking can explain the difference of recurrence since ethanol is thought of as a carcinogenic risk factor and would rather increase the recurrence risk of the flavonoid exposed patients.

Patient compliance with the flavonoid treatment was evaluated using information derived from a questionnaire given to 31 treated patients in the treatment group. 67% of these treated patients took the nutritional supplement longer than 12 mo, only 10% discontinued the intake within the first 3 mo. No side effects or unwanted symptoms were reported.

The habitual vegetable intake of the patients in both treatment and control groups (Tables 1 and 2) was rather low (< 3 d a week) and only about 40%-50% of the patients consumed vegetables \geq 3 d a week, which still is not sufficient for tumor prevention. About 16%-30% of the patients (cases and controls, Tables 1 and 2) reported low fruit content in their diet (< 3 d a week). Thus, no significant differences of the dietary habits were observed among treated and untreated patients. The self-administered questionnaire which was used to assess dietary habits provided only a crude estimate and was not validated; it is however a simple and practical tool that was well accepted and understood by the patients.

Flavonoids are part of human nutrition and are contained in vegetables and fruits, especially in apples, onions, berries, citrus fruits and teas but also in chocolate. Tea consumption of the patients was moderate and was

reported in most cases only as occasional tea drinking.

More patients with resected colorectal carcinoma of the control group (7 of 14, 50%) took aspirin compared to the cases (4 of 14, 28%) but this difference was not statistically significant (Table 2).

Surveillance by colonoscopy was performed in more cases (65%) than in controls (38%) and the time interval covered by colonoscopy was longer in treated patients than controls (Table 1). Thus the treated patients had a better chance for detection of neoplasia which would be a bias against a treatment effect. If the controls had more surveillance intensity, their recurrence rate would have been even higher.

In patients with prior adenomas that were removed by polypectomy and had surveillance colonoscopies, those treated with flavonoid treatment had a polyp recurrence rate similar to that of controls (about 50%). However, flavonoid treatment was associated with low risk incident adenomas while the control group included polyp recurrence of two adenomas with dysplasia (Figure 1). These differences were not statistically significant but could indicate that flavonoids could also suppress adenoma development and evolution. Cruz-Correa *et al* have recently reported that a combined treatment with quercetin (a flavonol) and curcumin (from curry) inhibited proliferation of adenomas in patients with familial adenomatous polyposis coli^[29]. These point to the possibility that flavonoids taken as long-term treatment could suppress neoplasia recurrence in high risk patients.

In conclusion, this pilot study which was controlled, prospective and observational, suggests that long-term flavonoid treatment could reduce the recurrence rate of colon neoplasia in high risk patients particularly in those with resected colorectal cancer. Therefore flavonoid supplementation should be investigated by further clinical studies to prove the efficacy and validity of this concept.

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COMMENTS

Background

Recurrence of cancer after a curative surgical resection in patients with colorectal cancer is a common problem that occurs in about 20%-40% depending on the previous tumor stage. It is essential for these patients to find ways to prevent this disaster.

Research frontiers

Prevention of recurrence can be achieved by adherence to a diet containing lots of fruits and vegetables or for higher tumor stages by cytostatic chemotherapy (adjuvant chemotherapy). Chemotherapy is very demanding and prone to unpleasant side effects. Dietary measures are difficult to implement and could give rise to bloating, gas and pain of the abdomen.

Innovations and breakthroughs

Other authors and articles seem to suggest that flavonoids could prevent colorectal cancers by healthy dietary habits, e.g. intake of foods with a high content of flavonoids. All these studies rely on epidemiological data and these are not always consistent and sometimes controversial. Our study uses an interventional approach with a nutritional supplement (as tablets) and this has not been done previously. Our data suggest that all patients at risk of recurrence of colorectal cancer should be treated with flavonoid supplements.

Peer review

It is a well-designed paper. The authors showed that sustained long-term treatment with a flavonoid mixture could reduce the recurrence rate of colon neoplasia in patients with resected colon cancer. This is an interesting article.

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RAPID COMMUNICATION

A red wine polyphenolic extract reduces the activation phenotype of cultured human liver myofibroblasts

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Author contributions: Neaud V performed the experiments; Rosenbaum J designed the study and wrote the paper.

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Abstract

AIM: To test the effect of a standardized red wine polyphenolic extract (RWPE) on the phenotype of human liver myofibroblasts in culture.

METHODS: Human myofibroblasts grown from liver explants were used in this study. Cell proliferation was measured with the 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) assay. Signaling events were analyzed by western blot with phospho-specific antibodies. Matrix-metalloproteinase activity was measured with gel zymography.

RESULTS: We found that cell proliferation was dose-dependently decreased by up to 90% by RWPE while cell viability was not affected. Exposure to RWPE also greatly decreased the phosphorylation of ERK1/ERK2 and Akt in response to stimulation by the mitogenic factor platelet-derived growth factor BB (PDGF-BB). Finally, RWPE affected extracellular matrix remodeling by decreasing the secretion by myofibroblasts of matrix-metalloproteinase-2 and of tissue inhibitor of matrix-metalloproteinases-1.

CONCLUSION: Altogether, RWPE decreases the activation state of liver myofibroblasts. The identification of the active compounds in RWPE could offer new therapeutic strategies against liver fibrosis.

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Key words: Liver fibrosis; Myofibroblasts; Hepatic stellate cells; Wine; Phosphorylation; Proliferation

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INTRODUCTION

Liver fibrosis is a serious health problem worldwide. It is a complication of most chronic liver diseases whether due to excessive alcohol consumption, chronic viral hepatitis B or C, non alcoholic steatohepatitis, hemochromatosis or others. The pathophysiology of liver fibrosis has been extensively studied (recently reviewed in^[1,2]). Whatever the initial insult, the abundant extracellular matrix (ECM) characteristic of liver fibrosis is synthesized by myofibroblastic cells. Myofibroblasts are mostly absent from the normal liver but at least two types of resident liver cells can be differentiated into myofibroblasts during liver disease: hepatic stellate cells, and portal fibroblasts^[3]. Myofibroblastic differentiation is characterized by a high rate of cell proliferation and of ECM synthesis and by cytoskeletal changes, notably expression of alpha smooth muscle actin (ASMA) that confers contractile properties to the cells^[4]. In addition, degradation of the normal liver ECM results from an increased secretion of the enzyme matrix metalloproteinase-2 (MMP-2) by myofibroblasts, while the proteolytic degradation of the abnormal ECM is inhibited due to a high level synthesis of a MMP inhibitor, tissue inhibitor of MMP-1 (TIMP-1)^[1,2]. In the recent years, a series of natural products were shown to be of potential benefit against liver fibrosis^[5,6]. For instance, we and others found that a polyphenolic component of red wine, trans-resveratrol, was able to strongly deactivate liver fibrogenic cells^[7,8], while a related molecule, trans-piceid, was ineffective^[8]. However, red wine contains many other polyphenolic substances, and red wine polyphenolic extracts (RWPE) showed many interesting biological effects in other settings, notably in the prevention of experimental atherosclerosis^[9]. One of the mechanisms postulated in this context is related to the inhibitory effect of RWPE on the proliferation of vascular smooth muscle cells^[10]. Since vascular smooth muscle cells share many characteristics with myofibroblasts, we tested the hypothesis that RWPE would affect the phenotypic characteristics of human liver myofibroblasts, especially those related to their pro-fibrogenic activity.

MATERIALS AND METHODS

Materials

Preparation and characterization of the polyphenolic extract (RWPE) from a red French wine (Corbières, A. O. C.) was as described previously^[11,12]. One liter of red wine produced 2.9 g of extract, which contained 471 mg/g total phenolic compounds expressed as gallic acid. Phenolic levels in the extract were obtained according to HPLC analysis procedure. In particular, the extract contained 8.6 mg/g catechin, 8.7 mg/g epicatechin, dimers (B1: 6.9 mg/g; B2: 8.0 mg/g; B3: 20.7 mg/g; and B4: 0.7 mg/g), anthocyanins (malvidin-3-glucoside: 11.7 mg/g; peonidin-3-glucoside: 0.66 mg/g; and cyanidin-3-glucoside: 0.06 mg/g), and phenolic acids (gallic acid: 5.0 mg/g; caffeic acid 2.5 mg/g; and caftaric acid: 12.5 mg/g). Stock solutions (1 mg/mL) were prepared in distilled water containing 1% ethanol and further diluted in culture medium. All dilutions were adjusted so as to contain 0.1% ethanol, and medium with 0.1% ethanol was used as a control.

Fetal calf serum (FCS) was from Dutscher (Brumath, France) and human serum from Etablissement Français du Sang (Bordeaux, France). Epidermal growth factor (EGF) was from Peprotech (Tebu, Le Perray en Yvelines, France) and recombinant platelet-derived growth factor BB (PDGF-BB) was from Eurobio (les Ulis, France). Phospho Thr308 AKT-1 and AKT-1 antibodies, phospho-ERK1/ERK2 and total ERK antibodies were from Cell Signaling Technology (Ozyme, Saint Quentin Yvelines, France). ASMA and vimentin antibodies were from Dako (Trappes, France). The TIMP antibody was from Santa Cruz Biotechnologies (Santa Cruz, CA). Beta-actin antibody was from Sigma (Saint Quentin Fallavier, France). IRDye 680 and IRDye 800 conjugated secondary antibodies; Odyssey Blocker and Odyssey infrared imaging system were from LI-COR Biosciences (ScienceTec, Les Ulis, France).

Cell isolation and culture

Human hepatic myofibroblasts were obtained from explants of non-tumor liver resected during partial hepatectomy and characterized as described previously^[13,14]. Specifically, the procedure, based on the selective growth advantage of myofibroblasts in the culture conditions used, allowed for 100% pure myofibroblasts population, as shown by positive staining for ASMA and vimentin, and negative staining for CD68 (a Kupffer cell marker), von Willebrand factor (an endothelial cell marker) or cytokeratin (an epithelial cell marker). This procedure is in accordance with INSERM ethical regulation imposed by French legislation. Myofibroblasts were used between the 3rd and the 6th passage, and were grown in DMEM containing 5% FCS, 5% pooled human serum and 5 ng/mL EGF. EGF was removed from the medium at least 3 d before experiments.

Cell proliferation assay

Cells were seeded at a density of 10^4 /well in 24-well plates. On the following day, the medium was replaced by DMEM with 5% FCS and RWPE dilutions to be tested. After three or seven days, the medium was removed and the cells

incubated with 1 mg/mL MTT [3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide] for 2 h at 37°C^[15]. The crystals were then dissolved with DMSO and the optical density was recorded at 540 nm. Results were expressed as proliferation index = (B-A)/(C-A) where A is the optical density recorded at d 0, B is the optical density with the test compound and C is the optical density obtained in the control wells.

Measure of cell DNA content

Cells were grown to confluence in 24-well plates, serum-starved for 24 h, then incubated with RWPE. After 24 h, the cells were lysed with NaH₂PO₄/Na₂HPO₄ 50 mmol/L, pH 7.4, NaCl 2 mol/L, EDTA 2 mmol/L, and DNA content was measured as described^[16].

Western blot

ERK and Akt phosphorylation was measured essentially as described previously^[17]. Briefly, cells were grown to confluency and serum-starved for three days. They were then pre-incubated with RWPE in serum-free Waymouth medium for 1 h, and then exposed to 20 ng/mL PDGF-BB for 10 min. At the end of the incubation, cell lysates were prepared in the presence of proteases and phosphatases inhibitors as described^[18]. Proteins were measured with a Bio-Rad assay. Equivalent amounts of proteins were separated by SDS-PAGE, transferred to polyvinylidene difluoride membranes, and analyzed by two color Western blotting with antibodies to total-ERK and phospho-ERK, or phospho-Akt-1 and β -actin. Blots were blocked in Odyssey Blocker and incubated simultaneously with both primary antibodies, followed by both IR-labeled secondary antibodies. Signals were detected and quantified using the Odyssey infrared imaging system.

The expression of ASMA and of vimentin was measured in cell extracts from cells exposed for seven days to RWPE using Western blot as described^[8].

The concentration of TIMP-1 in conditioned medium was also measured by Western blot. Confluent cells were incubated for two days with RWPE, the medium was collected, centrifuged and aliquots, normalized for DNA content of the cell layers, were analyzed by Western blot.

Zymography for MMP-2 activity

The detection of MMP-2 in conditioned medium was performed by gelatin zymography^[19], essentially as previously described^[8,20]. Briefly, cells were grown to confluence in 24-well plates, serum-starved for 24 h, then incubated with RWPE. The medium was collected, centrifuged and aliquots, normalized for DNA content of the cell layers, were analyzed on 8% SDS-PAGE gels containing 1 mg/mL gelatin. Following staining with Coomassie blue, MMP activity is detected as a white zone on a blue background.

Immunofluorescence detection of alpha-smooth muscle actin

Cells were seeded at a density of 10^4 /well on glass coverslips in 24-well plates. On the following day, the medium was replaced by DMEM with 5% FCS and RWPE

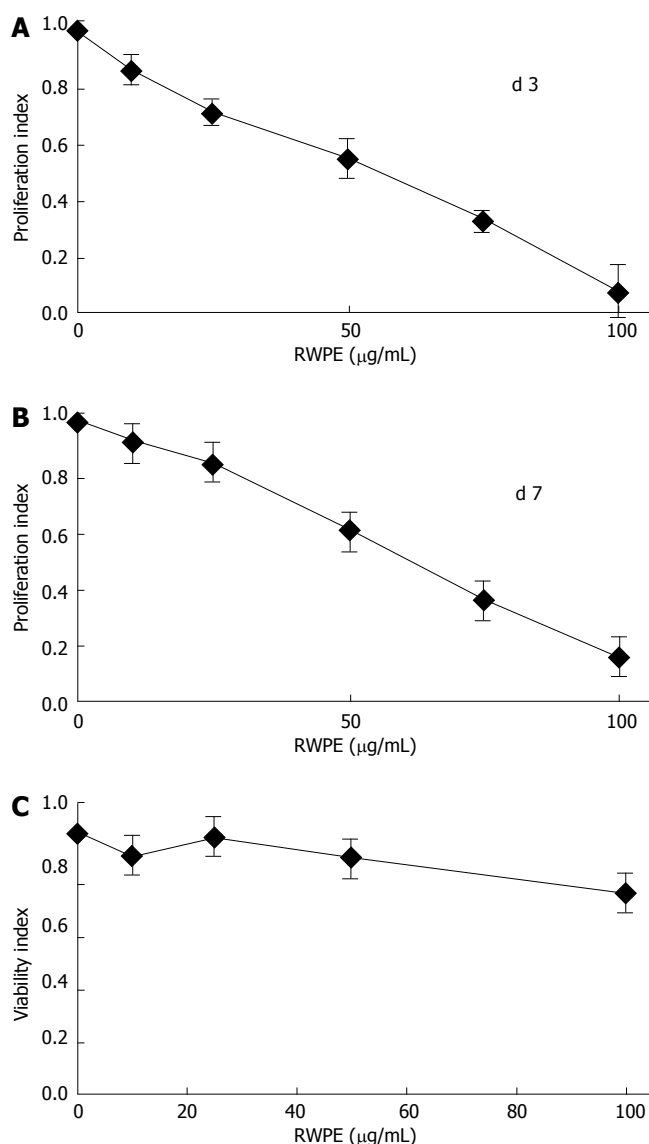


Figure 1 RWPE decreases myofibroblasts proliferation. Myofibroblasts were grown for either three (A) or seven days (B) in the presence of the indicated concentrations of RWPE. Results are the mean \pm SD of 4 independent experiments conducted in quadruplicate. The effect of RWPE was highly significant using ANOVA ($P < 0.0001$). In control experiments, myofibroblasts were exposed to RWPE for 24 h and the DNA content of the cell layer was measured (C). Results are expressed as the percentage of the values in treated cells as compared to cells treated with the solvent alone and are the mean \pm SD of 3 independent experiments conducted in quadruplicate. There were no significant differences between conditions.

dilutions to be tested. After three days, cells were fixed with methanol at -20°C , then incubated sequentially with an anti-ASMA monoclonal antibody and a Texas Red-conjugated secondary antibody. The slides were examined with a Zeiss Axioplan fluorescence microscope.

RESULTS

Effect of the RWPE on cell proliferation

The RWPE dose-dependently inhibited the proliferation of myofibroblasts down to $7.8\% \pm 4.5\%$ of control at d 3 and 15.8 ± 8.0 at d 7 (Figure 1A and B). The concentration that reduced growth by 50% was $50 \mu\text{g/mL}$. A toxic effect of RWPE on cells could be ruled out because no

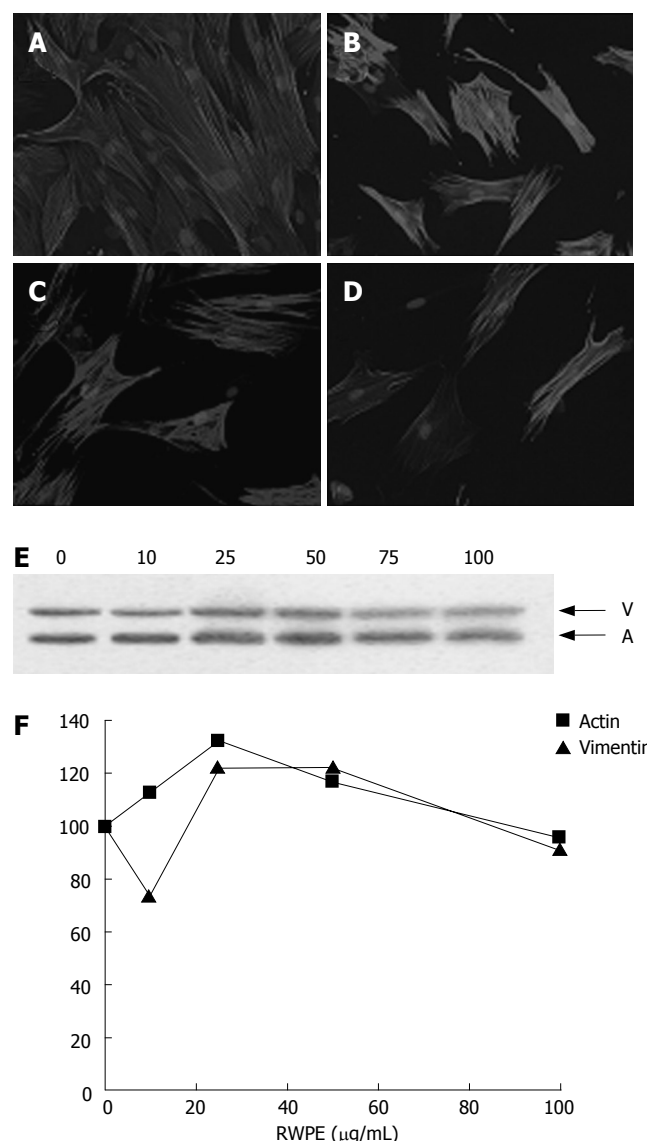


Figure 2 Effect of the RWPE on expression of alpha-smooth muscle actin. A: Myofibroblasts were incubated for seven days in the absence of RWPE (A) or with 50 (B), 75 (C), or 100 (D) $\mu\text{g/mL}$ RWPE. Aliquots of cell extracts grown in the same conditions were analyzed by Western blot (E) simultaneously for ASMA (A) and vimentin (V). F: The signals were quantified as described in Materials and Methods. The graph shows the mean of 2 separate experiments.

morphological signs of toxicity nor cell detachment were observed (Figure 2); furthermore, when confluent cells were exposed to a dose range of RWPE, there was no decrease in DNA content of the cell layers even at the highest concentrations (Figure 1C).

Effect of the RWPE on expression of alpha-smooth muscle actin

Expression of the cytoskeletal protein ASMA is hallmark of activated liver fibrogenic cells. We found that long term (up to seven days) exposure of liver myofibroblasts to RWPE did not affect ASMA expression. This was shown both by immunofluorescence and by Western blot (Figure 2). In addition, the expression of another cytoskeletal protein, vimentin, which expression is independent of fibrogenic cell activation, was also unaffected by RWPE treatment (Figure 2C).

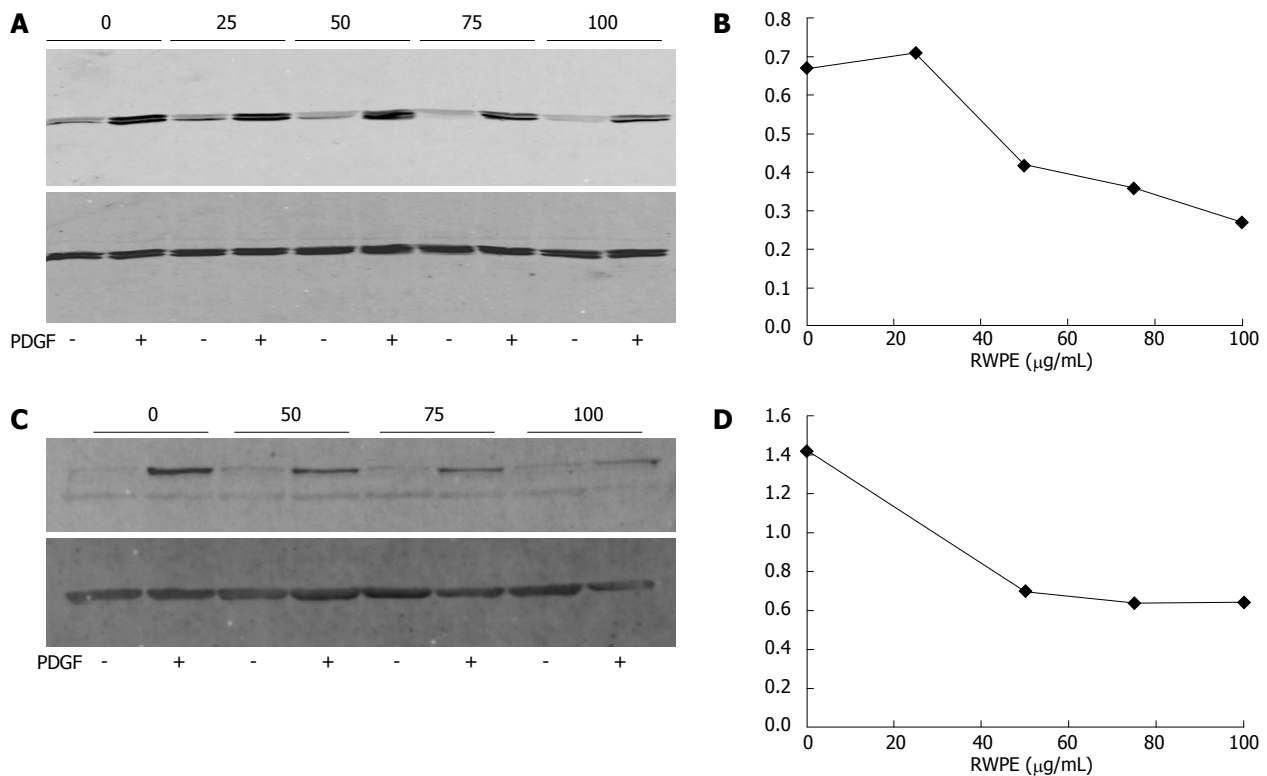


Figure 3 Effect of the RWPE on the phosphorylation of MAPK and Akt. **A:** Myfibroblasts were pre-incubated for 1 h with the indicated concentrations of RWPE (in μg/mL) or solvent, then exposed for 10 min to 20 ng/mL PDGF-BB or buffer. Identical amounts of cell extracts were analyzed by Western blot with antibodies to phospho-ERK1/ERK2 (top panel) and to total ERKs (bottom panel). The picture is representative of 3 experiments; **B:** Quantitative analysis of the experiment shown in (A). The activation index refers to the ratio between the levels of phospho-ERK to those of total ERK; **C:** Same as in A except that the blot was labelled with an anti-phospho-Akt antibody (top panel) and an antibody to β-actin (bottom panel); **D:** Quantitative analysis of the experiment shown in (C). The activation index refers to the ratio between the levels of phospho-Akt to those of β-actin.

Effect of the RWPE on the phosphorylation of MAPK and Akt

In order to delineate the mitogenic pathways affected by RWPE, myfibroblasts were briefly exposed to PDGF-BB. PDGF-BB is major mediator of liver fibrogenic cell activation, as it stimulates notably their proliferation^[13,21] and migration^[22,23], and is abundant in serum. We then examined the effect of RWPE on signalization pathways elicited by PDGF-BB. As expected, treatment with PDGF-BB induced a major increase in the phosphorylation of ERK1/ERK2 and of Akt. Exposure to RWPE greatly decreased the effect of PDGF-BB on the phosphorylation of both ERK1/ERK2 and Akt (Figure 3).

Effect of the RWPE on MMP-2 and TIMP-1 expression

A high level expression of the matrix remodeling enzyme MMP-2^[24], and of the inhibitor of extracellular matrix degradation, TIMP-1^[25], is characteristic of activated liver fibrogenic cells. Gelatin zymography showed a gelatinolytic band migrating at an apparent molecular weight of 72 kDa characteristic of MMP-2. The RWPE strikingly and dose-dependently decreased the secretion of MMP-2 (Figure 4A). It also greatly decreased TIMP-1 secretion, as assessed by Western blot (Figure 4B).

DISCUSSION

We show here that a standardized RWPE has striking effects on the phenotype of human liver myfibroblasts.

This is shown by a decreased proliferation rate together with decreased secretion of MMP-2 and of TIMP-1. These effects are not the consequence of a direct toxicity of the extract on cells as shown by morphological examination and DNA content measurement. We investigated further the mechanism of the decreased proliferation rate and found that RWPE treatment largely abolished the phosphorylation of ERK1/ERK2 and of Akt induced by the mitogenic factor PDGF-BB. Previously, Iijima *et al* showed that a RWPE decreased Akt but not ERK activation in vascular smooth muscle cells stimulated with PDGF^[26]. The differences may be due to the fact that different RWPEs were used, or to a true cell specificity. For instance, despite the fact that myfibroblasts and smooth muscle cells are related cells, we found that they had a differential response to trans-resveratrol^[8]. Some of the effects of the RWPE are reminiscent of those of trans-resveratrol, raising the possibility that RWPE effects may be due to this compound. However, resveratrol is present only at low concentration in wine (reviewed in^[27]) and is unlikely to explain the effects of RWPE. In addition, whereas it does indeed decrease myfibroblasts proliferation, MMP-2 secretion^[8] and Akt activation^[28], it does not affect ERK activation in response to PDGF^[28]. Furthermore, contrary to RWPE, it does decrease ASMA expression^[8].

The RWPE effects observed in the present study are potentially of benefit against liver fibrosis, if they held true in the *in vivo* situation. This seems obvious for the reduced cell proliferation that will decrease the number of ECM-

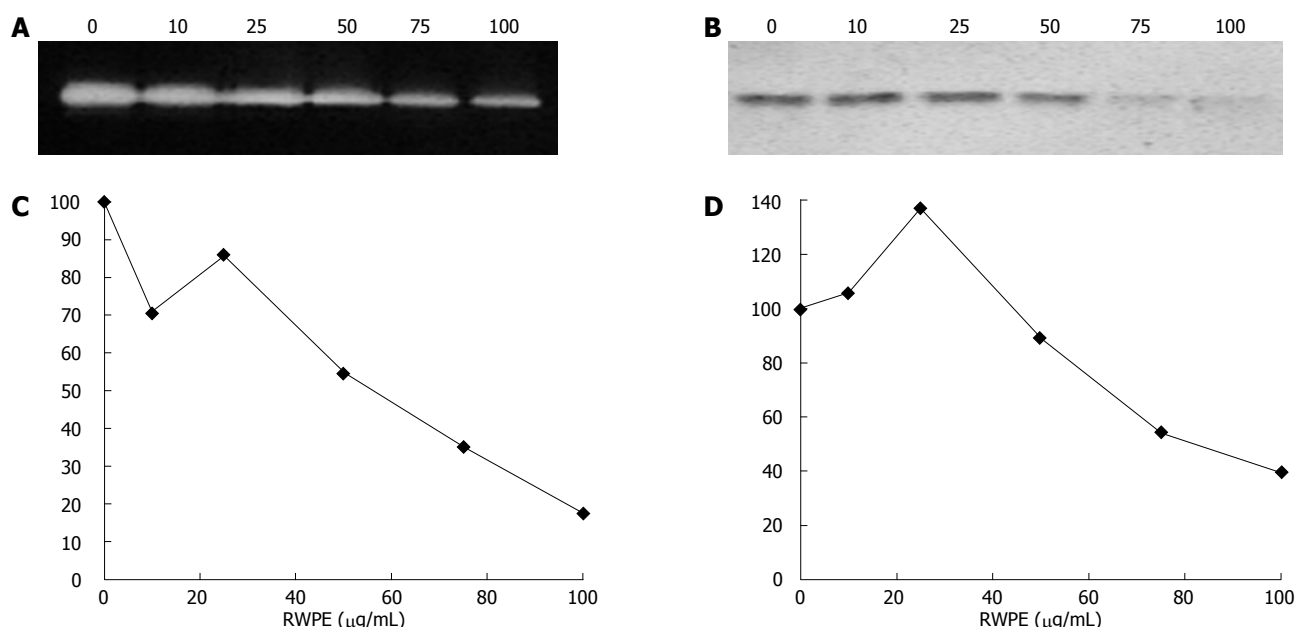


Figure 4 Effect of the RWPE on MMP-2 and TIMP-1 expression. **A:** Myfibroblasts were incubated for 24 h with the indicated concentrations of RWPE (in μg/mL). Aliquots of conditioned medium normalized for the DNA content of the cell monolayers were analyzed on gelatin-containing gels (**A**). The white bands on the dark background indicate gelatinolytic activity. Other aliquots were analyzed by Western blot with an antibody against TIMP-1 (**B**). Another experiment gave similar results; **C:** Quantitative analysis of the experiments. MMP-2: Mean of duplicate samples; TIMP-1: Mean of 2 independent experiments.

producing cells. Notably, the drastic effect on TIMP-1 secretion may have a major implication since TIMP-1 overexpression is considered one of the main determinants of liver fibrosis through the inhibition of ECM-degrading enzymes activity^[29,30].

Thus, although excessive wine consumption is one of the major causes of chronic liver diseases, wine itself may unexpectedly contain potent anti-fibrotic compounds. The identification of the active compounds in RWPE could offer new therapeutic strategies against liver fibrosis.

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We thank PL Teissedre for providing the standardized RWPE.

COMMENTS

Background

Liver fibrosis is a worldwide problem, as it complicates all chronic liver diseases. There is no established treatment for liver fibrosis.

Research frontiers

A series of natural products have shown beneficial effects on liver fibrosis in cell culture and animal models. In addition, red wine polyphenols were shown to reduce the proliferation of vascular smooth muscle cells, a cell type closely related to liver fibrogenic cells.

Innovations and breakthroughs

We found that a standardized red wine polyphenolic extract greatly decreased the proliferation of human liver fibrogenic cells. It also reduced their synthesis of matrix-metalloproteinase-2 and of the tissue inhibitor of matrix metalloproteinase-1, thus suggesting that it could affect the cell ability to remodel the extracellular matrix.

Applications

The identification of the active compound(s) in the extract could lead to *in vivo* testing of its anti-fibrotic activity in liver disease.

Terminology

Liver fibrosis: a common complication of most chronic liver diseases, where an excess of extracellular matrix components are deposited in the liver.

Peer review

This is a very nice communication and provides mechanistic data, is well-written, and ends with a thorough discussion. The results of this study are novel and potentially lay the ground work for the understanding of the effect of wine/wine extracts on human hepatic fibrosis.

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RAPID COMMUNICATION

Effects of estradiol and progesterone on the proinflammatory cytokine production by mononuclear cells from patients with chronic hepatitis C

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Abstract

AIM: To investigate the effects of estradiol (E2) and progesterone on the unstimulated and oxidative stress-stimulated production of tumor necrosis factor (TNF)- α , interleukin (IL)-1 β , IL-8, and macrophage chemotactic protein (MCP)-1 by peripheral blood mononuclear cells (PBMCs) from patients with chronic hepatitis C and healthy controls.

METHODS: The PBMCs were separated from age-matched 72 males and 71 females with and without chronic hepatitis C, who were divided into two groups based on a mean menopausal age of 50 years. Oxidative stress was induced by hydrogen peroxide in the cells incubated in serum-free media. Cytokines in the culture supernatant were measured by an enzyme-linked immunosorbent assay.

RESULTS: The highest levels of the spontaneous production of TNF- α , IL-1 β , IL-8, and MCP-1 by the unstimulated PBMCs were in the older male patients with chronic hepatitis C and the lowest levels were in the pre-

menopausal female healthy controls. E2 inhibited the cytokine production by the unstimulated PBMCs from the older male and post-menopausal female patients, which was further stimulated by progesterone. The exposure to hydrogen peroxide in the PBMCs from the younger male and pre-menopausal female healthy subjects induced the production of cytokines. The change rates of the hydrogen peroxide-stimulated cytokine production were suppressed by E2 and enhanced by progesterone.

CONCLUSION: These findings suggest that E2 may play a favorable role in the course of persistent liver injury by preventing the accumulation of monocytes-macrophages and by inhibiting proinflammatory cytokine production, whereas progesterone may counteract the favorable E2 effects.

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Key words: Estradiol; Progesterone; Mononuclear cell; Proinflammatory cytokine; Chemokine

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INTRODUCTION

In inflammatory and oxidative liver injury, the accumulation of leukocytes and macrophages including Kupffer cells on the sites of injury and inflammation in the liver is thought to be mediated by such cytokines as chemokines, including interleukin (IL)-8 and macrophage chemotactic protein (MCP)-1. These monocytes and macrophages are in turn

able to release proinflammatory cytokines such as tumor necrosis factor (TNF)- α and IL-1 β , thus leading to the occurrence of persistent liver injury. It has been reported that chronic hepatitis C virus (HCV) infection tends to progress more rapidly in men than in women^[1,2], and that the decline in estradiol (E2) production with menopause is associated with a spontaneous increase in TNF- α and IL-1 β ^[3]. It should be noted that large increases in the amount of reactive oxygen species (ROS) lead to disturbance of prooxidant-antioxidant balance, or oxidative stress. Our previous studies have shown that hepatocytes possessed estrogen receptor (ER), and that in cultured hepatocytes in a state of oxidative stress, E2 inhibited the activation of the nuclear factor κ B (NF- κ B), a key transcription factor that induces multiple genes in response to inflammation and oxidative stress^[4], through ER^[5,6]. These findings suggest that E2 could enhance the anti-inflammatory activity in the injured liver by decreasing the proinflammatory cytokine production, and it might, therefore, play a cytoprotective role through ER in the liver^[7,8]. However, regarding E2 and another female sex steroid, progesterone, there is little information about the direct effects of these sex hormones on the production of chemokines and proinflammatory cytokines by monocytes and macrophages in chronic HCV infection, although *in vitro* experiments in which monocytes were incubated with sex hormones have revealed conflicting results regarding monocyte cytokine production^[9]. Therefore, this study investigated the effects of E2 and progesterone on the production of TNF- α , IL-1 β , IL-8, and MCP-1 by the heparinized peripheral blood mononuclear cells (PBMCs), after stimulation with the ROS, hydrogen peroxide^[10]. These effects were then compared between the blood samples from the age-matched male and female patients with chronic hepatitis C and the healthy controls, which were divided into two groups based on a mean menopausal age of 50 years.

MATERIALS AND METHODS

Patients

This study was conducted at Tokushima University Hospital and the subjects consisted of age-matched males ($n = 72$) and females ($n = 71$) including 71 patients with chronic hepatitis C and 72 healthy controls who were divided into younger, or pre-menopausal (< 50 years of age), and older, or post-menopausal (≥ 50 years of age) groups. The chronic hepatitis patients met the following criteria: a persistently elevated serum alanine aminotransferase (ALT, normal serum levels of ALT range between 5 and 40 U/L) level for at least six months; HCV-RNA seropositivity; seronegativity for Hepatitis B virus (HBV) surface antigen; exclusion of all other potential cause of chronic liver disease such as autoimmune hepatitis, primary biliary cirrhosis, drug-induced hepatitis, or metabolic liver disease; and no history of alcohol abuse, defined as an alcohol intake of more than 20 g per day over the previous year. In addition, any individuals taking oral contraceptives or corticosteroids, who had previously undergone an operation on the ovaries were also excluded from the study.

All females in the younger groups with and without

chronic HCV infection had a normal ovarian cycle, and the mean ovarian cycle length was 28 d with a range of 26 to 32 d. Ethical approval was obtained from the Tokushima University Hospital ethics committee, and informed consent was obtained from all patients taking part in the study.

The heparinized peripheral blood samples were obtained in the morning after an overnight fast. In the pre-menopausal females, the blood samples were taken during the luteal phase 5 to 8 d before the onset of menses. The human PBMC fractions were separated using density-gradient centrifugation with a lymphocyte separation medium (Organon Teknika, Durham, NC, USA). After three washes with phosphate-buffered saline and buffered RPMI 1640 supplemented with 100 U/mL penicillin, 100 μ g/mL streptomycin, and 1% L-glutamine (RPMI culture medium), the mononuclear cells were suspended at a concentration of 1×10^6 /mL in RPMI culture medium with 10% heat-inactivated fetal bovine serum. The cells were cultured at 37°C in a 5% CO₂ atmosphere and 100% humidity for 24 h and then treated with and without 17 β -E2 (Sigma, St Louis, MO, USA) or progesterone (Wako, Osaka, Japan) for another 6 h in the presence and absence of the estrogen receptor antagonist, ICI-182, 780 (ICI: Tocris Cookson, Ballwin, MO, USA), or the PR antagonist RU486 (RU: Wako). In another experiment, the culture medium was changed with serum-free RPMI, and oxidative stress was induced by hydrogen peroxide (10^{-7} - 10^{-5} mol/L). The cells were then incubated with either E2 or progesterone for up to 6 h in the presence and absence of ICI or RU. The steroid sex hormones and receptor antagonists were initially prepared as an ethanol stock solution (10^{-2} mol/L) and then were diluted with the culture medium in order to obtain an appropriate working solution concentration.

Cytokine and female sex hormone assays

The cytokines of TNF- α , IL-1 β , IL-8 and MCP-1 and the female sex hormones of E2 and progesterone secreted into the culture supernatant were measured by an enzyme-linked immunosorbent assay (ELISA) using commercial kits (R&D Systems, Minneapolis, MN, USA) and by a radioimmunoassay using commercial kits (CIS Diagnostic, Tokyo, Japan), respectively, according to the manufacturer's instructions.

Statistical analysis

The data were presented as the mean \pm SD, unless otherwise indicated. The means were compared between the two groups using the Wilcoxon's signed-rank test and the Mann-Whitney *U* test with Bonferroni correction. A *P* value of less than 0.05 was considered to be statistically significant.

RESULTS

Characteristics at the time of isolation of PBMCs from age-matched younger and older males and pre- and post-menopausal females of patients with chronic hepatitis C and healthy controls

As shown in Table 1, the background factors of 4 patient groups with chronic hepatitis C, such as the serum levels of

Table 1 Characteristics at the time of isolation of PBMCs from age-matched younger and older males and pre- and post-menopausal females of patients with chronic hepatitis C and healthy controls

	Patients with chronic hepatitis C				Healthy controls			
	Males (yr)		Females (yr)		Males (yr)		Females (yr)	
	< 50	≥ 50	< 50	≥ 50	< 50	≥ 50	< 50	≥ 50
<i>n</i>	18	18	17	18	18	18	18	18
Age (yr)	41 ± 7	69 ± 6	41 ± 7	68 ± 6	40 ± 6	68 ± 6	40 ± 6	69 ± 6
HCV-RNA (log copies/mL)	6.1 ± 0.8	6.5 ± 1.4	5.9 ± 0.8	6.4 ± 1.2	ND	ND	ND	ND
ALT (U/L)	118 ± 68	112 ± 52	105 ± 49	101 ± 55	27 ± 8	26 ± 8	24 ± 7	24 ± 8
Platelet count (10 ³ /mm ³)	208 ± 58	189 ± 52	206 ± 54	185 ± 55	230 ± 60	219 ± 53	225 ± 57	214 ± 58

The PBMCs were isolated from age-matched males (*n* = 72) and females (*n* = 71) of 71 patients with chronic hepatitis C and 72 healthy controls, who were divided into younger or pre-menopausal (< 50 years of age) and older or post-menopausal (≥ 50 years of age) groups, based on a mean menopausal age of 50 years. The values are the mean ± SD (*n* = 17 or 18). ND: Not detected.

Table 2 Spontaneous production of TNF-α, IL-1β, IL-8, and MCP-1 by PBMCs from age-matched younger and older males and pre- and post-menopausal females of patients with chronic hepatitis C and healthy controls

Subjects with and without chronic HCV infection			TNF-α	IL-1β	IL-8	MCP-1
			(pg/mL supernatant)			
Males	Younger (< 50 yr)	Patients	354 ± 201 ^a	253 ± 142	918 ± 499	403 ± 216
		Controls	265 ± 161	180 ± 105	780 ± 420	308 ± 161
	Older (≥ 50 yr)	Patients	385 ± 234 ^a	284 ± 160 ^a	1087 ± 576 ^a	461 ± 242 ^a
		Controls	322 ± 200	217 ± 125	899 ± 480	364 ± 187
Females	Pre-menopausal (< 50 yr)	Patients	288 ± 177	198 ± 111	780 ± 410	334 ± 174
		Controls	210 ± 126	167 ± 94	624 ± 335	275 ± 140
	Post-menopausal (≥ 50 yr)	Patients	314 ± 189	264 ± 149 ^a	887 ± 473	387 ± 198
		Controls	241 ± 148	182 ± 100	699 ± 372	362 ± 201

The PBMCs were isolated from age-matched males (*n* = 72) and pre- and post-menopausal females (*n* = 71) of 71 patients with chronic hepatitis C and 72 healthy controls. The levels of TNF-α, IL-1β, IL-8, and MCP-1 in the culture supernatant were detected by means of an ELISA. The values are the mean ± SD (*n* = 17 or 18). ^a*P* < 0.05 vs the pre-menopausal healthy controls.

ALT and HCV-RNA, and platelet counts (normal ranges between 150 and 350 × 10³/mm³), were not significantly different among the younger (< 50 years of age), or pre-menopausal, and the older (≥ 50 years of age), or post-menopausal groups. There was no significant difference of platelet counts between the patient groups and healthy control groups. Because platelet counts in chronic hepatitis C patients have been reported to be an indicator of the degree of hepatic fibrosis^[11], most of the patients in this study seemed to show mild hepatic fibrosis.

Comparison of the spontaneous production of TNF-α, IL-1β, IL-8, and MCP-1 by PBMCs from age-matched younger and older males and pre- and post-menopausal females of patients with chronic hepatitis C and healthy controls

PBMCs were isolated from age-matched younger and older males and pre- and post-menopausal females of the patients with chronic hepatitis C and the healthy controls. In the premenopausal females, the blood samples were taken during the luteal phase of the ovarian cycle. The 24-h cultured PBMCs released TNF-α, IL-1β, IL-8, and MCP-1 into the culture medium (Table 2). The levels of E2 and progesterone in the culture supernatant were found to be under 10⁻¹² mol/L. Although the net cytokine levels were considerably different among the individuals, the spontaneous production levels of cytokines in the unstimulated PBMCs appeared to show different tendencies between the 8 subgroups, the highest levels

were present in the older male patients and the lowest levels were found in the pre-menopausal female controls (*P* = 0.039 for TNF-α, *P* = 0.018 for IL-1β, *P* = 0.013 for IL-8, and *P* = 0.011 for MCP-1). The chronic hepatitis C patients showed higher production levels of TNF-α, IL-1β, IL-8, and MCP-1 as compared with the age-matched healthy controls, and the mean production levels of cytokines in the older groups were higher than those in the younger groups, while the female subjects tended to produce much less cytokines from the unstimulated PBMCs than did the age-matched male subjects (Table 2).

Effects of E2 and progesterone on spontaneous production of TNF-α, IL-1β, IL-8, and MCP-1 by unstimulated PBMCs from age-matched older male and post-menopausal female patients with chronic hepatitis C

We next investigated the effects of E2 and progesterone on the augmented production of TNF-α, IL-1β, IL-8, and MCP-1 by the unstimulated PBMCs from the age-matched older male and post-menopausal female patients with chronic hepatitis C. When the unstimulated PBMCs were cultured for another 6 h without female sex hormones or receptor antagonists, the mean percentages of each initial value for the cytokine production reached up to 110%-123% (Table 3). Whereas the treatment with E2 and progesterone in the unstimulated PBMCs for 6 h significantly affected the change rates of the cytokine production in a dose dependent manner, the percentages

Table 3 Effects of E2 and progesterone on spontaneous production of TNF- α , IL-1 β , IL-8, and MCP-1 by unstimulated PBMCs from age-matched older male and post-menopausal female patients with chronic hepatitis C

Chronic hepatitis C subjects	TNF- α	IL-1 β	IL-8	MCP-1
			(% of initial value)	
Older male patients				
None	123 \pm 14	120 \pm 16	119 \pm 12	114 \pm 15
+ E2	82 \pm 10 ^a	84 \pm 10 ^a	80 \pm 11 ^a	78 \pm 11a
+ E2 + ICI	118 \pm 16	114 \pm 18	108 \pm 19	99 \pm 19
+ Progesterone	167 \pm 19 ^a	169 \pm 20 ^a	159 \pm 18 ^a	169 \pm 28 ^a
+ Progesterone + RU	129 \pm 21	124 \pm 21	132 \pm 19	122 \pm 24
Post-menopausal female patients				
None	118 \pm 15	119 \pm 14	122 \pm 16	110 \pm 13
+ E2	83 \pm 11 ^a	79 \pm 9 ^a	81 \pm 12 ^a	74 \pm 11 ^a
+ E2 + ICI	108 \pm 14	122 \pm 16	117 \pm 19	104 \pm 19
+ Progesterone	167 \pm 13 ^a	170 \pm 24 ^a	159 \pm 20 ^a	175 \pm 30 ^a
+ Progesterone + RU	130 \pm 27	125 \pm 28	132 \pm 30	123 \pm 25

The unstimulated PBMCs from the age-matched older male and post-menopausal female patients with chronic hepatitis C were cultured for another 6 h with and without 10^{-8} mol/L E2 (E2) or 10^{-7} mol/L progesterone (progesterone) in the presence and absence of 10^{-6} mol/L ICI (ICI) or 10^{-6} mol/L RU (RU). The levels of TNF- α , IL-1 β , IL-8, and MCP-1 in the culture supernatant were detected by means of an ELISA. The results were expressed as the percentages of each initial value for the cytokine production in the absence of the female sex hormones and receptor antagonists. The values are the mean \pm SD ($n = 18$). ^a $P < 0.05$ vs 6-h-cultures in the absence of the female sex hormones and receptor antagonists.

of each initial value decreased significantly in the PBMCs treated with E2 at 10^{-8} and 10^{-7} mol/L, and they increased significantly in the PBMCs treated with 10^{-7} mol/L progesterone (Figure 1). There was no significant difference between the change rates of the cytokine production in the male and female patients with chronic hepatitis C.

The inhibitory effects of 10^{-8} mol/L E2 on the unstimulated cytokine production in the male and female patients were blocked by the specific ER antagonist ICI at a dose of 10^{-6} mol/L, while the further enhancement effects of 10^{-7} mol/L progesterone on the unstimulated cytokine production in both genders were blocked by the PR antagonist RU (Table 3). The treatment with ICI or RU alone had no effect on any of the parameters examined herein (data not shown).

Effects of E2 and progesterone on hydrogen peroxide-stimulated production of TNF- α , IL-1 β , IL-8, and MCP-1 by PBMCs from age-matched younger male and pre-menopausal female healthy controls

The exposure to low doses of hydrogen peroxide (10^{-7} – 10^{-5} mol/L) in the PBMCs from the age-matched younger male and pre-menopausal female healthy controls, incubated in serum-free RPMI for 6 h, was observed to stimulate the production of TNF- α , IL-1 β , IL-8, and MCP-1 in a dose-dependent manner (data not shown). The subsequent studies used a dose of 10^{-5} mol/L of hydrogen peroxide for further stimulation of the incubated PBMCs. The exposure to hydrogen peroxide induced a time-dependent and transient cytokine production, peaking at 1–6 h, over a 6 h period (Figure 2). There was no significant difference between the cytokine levels in the male and female healthy controls. The cytokine levels in the culture supernatant (Figure 2) peaked after 6 h. Subsequent studies used an incubation time of 6 h to measure the levels of TNF- α , IL-1 β , IL-8, and MCP-1 after the hydrogen peroxide exposure.

The hydrogen peroxide-stimulated cytokine production was inhibited by 10^{-8} mol/L E2 (Table 4). The inhibitory effect of E2 was blocked by 10^{-6} mol/L ICI (Table 4). In contrast to E2, progesterone treatment for 6 h resulted in the further cytokine production in the oxidative stress-stimulated PBMCs. The stimulatory effect of progesterone (10^{-7} mol/L) was blocked by 10^{-6} mol/L RU (Table 4). No parameters examined in the PBMCs were found to be significantly different between the male and female subjects.

DISCUSSION

In the present study, the highest levels of the spontaneous production of TNF- α , IL-1 β , IL-8, and MCP-1 by the unstimulated PBMCs were found to be in the older male patients with chronic hepatitis C, and the lowest levels were in the pre-menopausal female healthy subjects, although the cytokine levels were considerably different among the individuals. The male subjects tended to produce cytokines from the unstimulated PBMCs to a much greater degree than did the age-matched female subjects. The augmented cytokine production by the PBMCs from the older male and post-menopausal female patients with chronic hepatitis C was inhibited by supplementation with E2, and was further stimulated by supplementation with progesterone through their receptors, when the unstimulated cells were cultured for an additional 6 h. The exposure to low doses of hydrogen peroxide in the PBMCs from younger male and pre-menopausal female healthy subjects incubated in the serum-free media for 6 h was observed to induce cytokine production. The change rates of the hydrogen peroxide-stimulated production of TNF- α , IL-1 β , IL-8, and MCP-1 in the PBMCs were suppressed by E2, and were enhanced by progesterone through their receptors. The specificity of the E2-mediated anti-inflammatory induction through the ER and the progesterone-mediated proinflammatory induction through the PR was shown

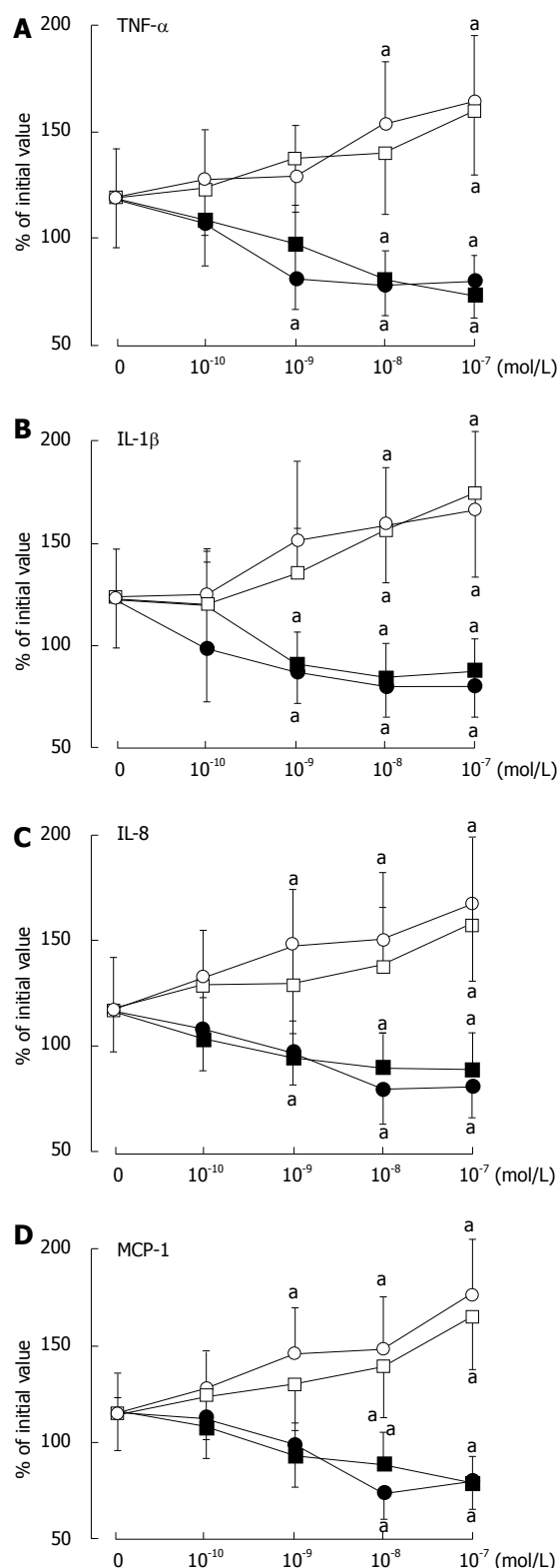


Figure 1 Effects of E2 and progesterone on spontaneous production of TNF- α (A), IL-1 β (B), IL-8 (C), and MCP-1 (D) by unstimulated PBMCs from age-matched older male and post-menopausal female patients with chronic hepatitis C. The unstimulated PBMCs from the age-matched older male (black square) and post-menopausal female (black circle) patients with chronic hepatitis C were cultured for another 6 h with and without E2 (10^{-10} - 10^{-7} mol/L) (solid) or progesterone (10^{-10} - 10^{-7} mol/L) (open). The spontaneous production levels of TNF- α (A), IL-1 β (B), IL-8 (C), and MCP-1 (D) in the culture supernatant were detected by means of an ELISA. The results were expressed as the percentages of each initial value for the cytokine production in the absence of the female sex hormones. The values are the mean \pm SD ($n = 18$). $^aP < 0.05$ in comparison to the 6-h-cultures in the absence of the female sex hormones.

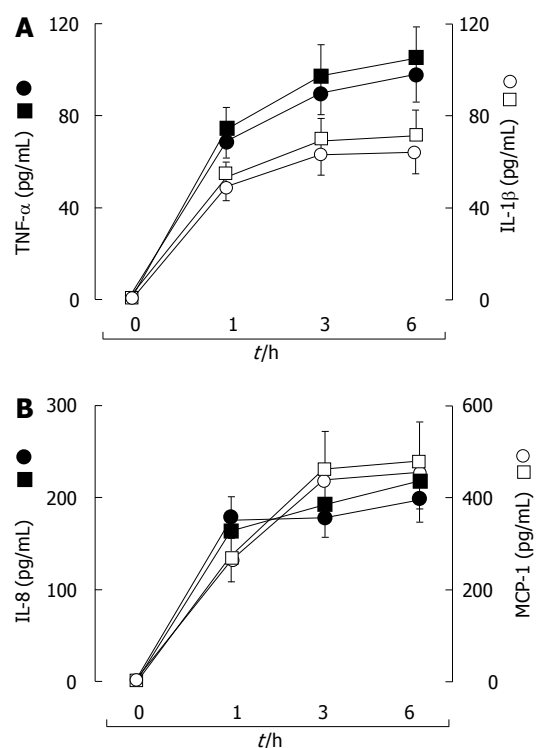


Figure 2 Stimulation of TNF- α , IL-1 β (A), IL-8, and MCP-1 (B) production after exposure to hydrogen peroxide by unstimulated PBMCs from age-matched younger male and pre-menopausal female healthy controls. The unstimulated PBMCs from the age-matched younger male (black square) and pre-menopausal female (black circle) healthy controls were incubated for up to 6 h in serum-free RPMI in the presence of 10^{-5} mol/L hydrogen peroxide. The levels of TNF- α (A solid), IL-1 β (A open), IL-8 (B solid), and MCP-1 (B open) in the culture supernatant were detected by means of an ELISA. The values are the mean \pm SD ($n = 10$).

by ICI and RU, respectively, in both the unstimulated and oxidative stress-stimulated PBMCs. The inhibitory effect of E2 at a dose of 10^{-8} mol/L on the unstimulated and stimulated cytokine production was blocked by ICI in both gender subjects. Treatment with the progesterone receptor antagonist RU led to a blockage of further cytokine production induced with 10^{-7} mol/L progesterone by the unstimulated and stimulated PBMCs from both genders. No parameters examined in the PBMCs were found to be significantly different between the male and female subjects.

There is a large body of evidence indicating that the decline in the ovarian function with menopause is associated with spontaneous increases in TNF- α , IL-1 β , and IL-6^[3]. E2, at physiological concentrations (10^{-11} - 10^{-8} mol/L), has been reported to inhibit the spontaneous secretion of these proinflammatory cytokines in whole blood cultures^[12] or PBMCs^[13]. The unstimulated production of TNF- α and IL-1 β in PBMCs has been reported to be higher in patients with chronic hepatitis C than in healthy subjects^[14]. These findings were consistent with the present data. The *in vivo* treatment with E2 transdermally in postmenopausal women has been reported to decrease the spontaneous IL-6 production by PBMCs after 12 mo of the therapy^[13]. One preliminary study also showed the hydrogen peroxide-induced TNF- α

Table 4 Effects of E2 and progesterone on hydrogen peroxide-stimulated production of TNF- α , IL-1 β , IL-8, and MCP-1 by PBMCs from age-matched younger male and pre-menopausal female healthy controls

Healthy subjects	TNF- α	IL-1 β (pg/mL supernatant)	IL-8	MCP-1
Younger male controls				
Oxidative stress	102 \pm 17	66 \pm 11	218 \pm 41	474 \pm 81
Oxidative stress + E2	78 \pm 11 ^a	49 \pm 6 ^a	174 \pm 27 ^a	332 \pm 49 ^a
Oxidative stress + E2 + ICI	107 \pm 10	62 \pm 11	223 \pm 46	457 \pm 87
Oxidative stress + Progesterone	140 \pm 14 ^a	86 \pm 9 ^a	289 \pm 40 ^a	658 \pm 98 ^a
Oxidative stress + Progesterone + RU	110 \pm 23	75 \pm 14	223 \pm 32	516 \pm 73
Pre-menopausal female controls				
Oxidative stress	91 \pm 15	57 \pm 9	195 \pm 37	435 \pm 85
Oxidative stress + E2	64 \pm 11 ^a	42 \pm 6 ^a	155 \pm 29 ^a	314 \pm 68 ^a
Oxidative stress + E2 + ICI	95 \pm 13	54 \pm 9	187 \pm 37	419 \pm 92
Oxidative stress + Progesterone	129 \pm 12 ^a	75 \pm 12 ^a	255 \pm 40 ^a	586 \pm 70 ^a
Oxidative stress + Progesterone + RU	101 \pm 19	63 \pm 11	208 \pm 40	462 \pm 73

The unstimulated PBMCs from the age-matched younger male ($n = 18$) and pre-menopausal female ($n = 18$) healthy controls were incubated for up to 6 h in serum-free RPMI after exposure to 10^{-5} mol/L hydrogen peroxide (oxidative stress) with and without 10^{-8} mol/L E2 (E2) or 10^{-7} mol/L progesterone (Progesterone) in the presence and absence of 10^{-6} mol/L ICI (ICI) or 10^{-6} mol/L RU (RU). The levels of TNF- α , IL-1 β , IL-8, and MCP-1 in the culture supernatant incubated for 6 h were detected by means of an ELISA. The values are the mean \pm SD ($n = 18$). ^a $P < 0.05$ vs 1 h cultures for MCP-1 or 6 h cultures for TNF- α , IL-1 β , and IL-8 after hydrogen peroxide exposure in the absence of the female sex hormones and receptor antagonists.

and MCP-1 expressions to be attenuated by E2 in the peritoneal macrophages of female mice^[15]. Furthermore, E2 is able to attenuate IL-1 β in ER expressing HepG2 cells^[16], and to ameliorate the burn-induced increase in the serum TNF- α levels in rats^[17]. These findings suggest that E2 may exert a hepatoprotective action against inflammation and oxidative stress, at least in part, by preventing accumulation of monocytes and macrophages and by inhibiting the production of proinflammatory cytokines.

As far as the *in vitro* studies with female sex hormones on the production of TNF- α and IL-1 β by monocytes are concerned, however, conflicting data have been published^[9], varying from some^[18-20] to no^[12,21] effect of E2 or progesterone on cytokine production. E2 has been reported to suppress the TNF- α production in unstimulated PBMCs, but not in endotoxin-stimulated PBMCs, from postmenopausal females with osteoporosis^[22]. An inhibition of IL-1 β production in endotoxin-stimulated monocytes by E2 or progesterone at physiological concentrations has also been reported^[23]. The results of the reported studies did not correlate with the present data. These conflicting results may possibly be due to the handling of the cells during the *in vitro* research, different experimental methods used, and/or differences in the subjects employed in the studies.

In the premenopausal female subjects with and without chronic hepatitis C enrolled herein, the blood samples were taken during the luteal phase of the menstrual cycle. During the luteal phase, the serum concentration of endogenous progesterone rises up to a maximum of about 10^{-7} mol/L, which can be ten to a hundred times higher than E2. Higher blood levels of TNF- α have been observed during the luteal phase in comparison to the follicular phase^[24]. In males, a higher percentage of IL-1 β producing stimulated monocytes has been demonstrated in comparison to females in the follicular phase^[19]. The male sex hormone testosterone has some structural and functional similarities to

progesterone^[25]. Judging from these findings and the present data showing that treatment with E2 (10^{-8} and 10^{-7} mol/L) and progesterone (10^{-7} mol/L) significantly affected the change rate of the cytokine production in hydrogen peroxide-stimulated PBMCs, E2 may, therefore, exert an anti-inflammatory action against both inflammation and oxidative stress in the mononuclear cells from the chronic hepatitis C patients, whereas progesterone may counteract the favorable effects of E2.

HCV infections are recognized to be a major causative factor in the development of liver injury leading to cirrhosis^[26,27]. The HCV core protein has been reported to enhance the signaling pathway of NF- κ B activation in human hepatoma HuH-7 and cervical cancer HeLa cells, and the HCV core protein is triggered by TNF- α -related cytokines^[28]. Damage to the parenchymal cell membranes and liver mitochondria could produce ROS derived from lipid peroxidative processes, which constitute a general feature of a sustained inflammatory response and liver injury^[29]. In comparison to other types of ROS, hydrogen peroxide is more stable and membrane permeable leading to the hypothesis that it acts as a second messenger in regulating the signaling events, including the mitogen-activated protein kinase (MAPK) activation. We have already reported that E2 inhibited the prooxidant-induced lipid peroxidation in rat liver mitochondria^[5], attenuated ROS generation and NF- κ B activation in cultured rat hepatocytes in a state of prooxidant-induced oxidative stress^[6], while also suppressing the hydrogen peroxide-induced activation of MAPKs and transcription factors including NF- κ B in cultured rat hepatic stellate cells^[30]. In the present study, hydrogen peroxide exposure resulted in an increase in the TNF- α , IL-1 β , IL-8, and MCP-1 levels in the cultured mononuclear cells from the male and female healthy subjects. The oxidative stress-stimulated cytokine expression was attenuated by E2 and augmented by progesterone in a dose-dependent manner without any significant difference between the males and females. These effects of E2 and progesterone were blocked by

their receptor antagonists ICI and RU, indicating that ER and PR could mediate female sex hormone action in the oxidative stress-stimulated monocytes and macrophages.

Finally, the current data suggest that E2 may play a favorable role in the course of persistent liver injury, at least in part, by preventing the accumulation of monocytes and macrophages and by also inhibiting the proinflammatory cytokine production through ER, whereas progesterone may counteract these positive E2 effects by enhancing the accumulation of inflammatory cells and their cytokine production through PR.

COMMENTS

Background

Parenchymal cell membrane damage could produce reactive oxygen species (ROS) derived from lipid peroxidative processes, which represent the general feature of sustained inflammatory response and liver injury. A chronic hepatitis C virus infection tends to progress more rapidly in men than women. There is little information about the effects of estradiol (E2) and progesterone on the proinflammatory cytokine production by prooxidant-stimulated monocytes in chronic hepatic C patients.

Research frontiers

We have recently hypothesized that E2 could play a cytoprotective role in the injured liver by inhibiting the ROS generation, antioxidant enzyme loss, and induction of redox sensitive transcription factors.

Innovations and breakthroughs

This study focused on comparison of the oxidative stress-stimulated proinflammatory cytokine production between younger and older patients, who were divided into two groups based on a mean menopausal age of 50 years, demonstrating that E2 might play a favorable role in the course of persistent liver injury, whereas progesterone might counteract the favorable E2 effects.

Applications

The favorable activity of E2 may be involved in the persistent liver injury of different liver diseases such as alcoholic and non-alcoholic liver diseases as well as chronic hepatitis C and B.

Peer review

The study is of particular interest because the role and importance of the sex hormones in the regulation of some cell functions during disturbance in the prooxidant-antioxidant balance is understood. It is very important that study links clinical and experimental part.

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RAPID COMMUNICATION

Comparison of CT and MRI for presurgical characterization of paraaortic lymph nodes in patients with pancreatico-biliary carcinoma

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Abstract

AIM: To determine the accuracy of computed tomography (CT) and magnetic resonance (MR) for presurgical characterization of paraaortic lymph nodes in patients with pancreatico-biliary carcinoma.

METHODS: Two radiologists independently evaluated CT and MR imaging of 31 patients who had undergone lymphadenectomy (9 metastatic and 22 non-metastatic paraaortic nodes). Receiver operating characteristic (ROC) curve analysis was performed using a five point scale to compare CT with MRI. To re-define the morphologic features of metastatic nodes, we evaluated CT scans from 70 patients with 23 metastatic paraaortic nodes and 47 non-metastatic ones. The short axis diameter, ratio of the short to long axis, shape, and presence of necrosis were compared between metastatic and non-metastatic nodes by independent samples *t*-test and Fisher's exact test. $P < 0.05$ was considered statistically significant.

RESULTS: The mean area under the ROC curve for CT (0.732 and 0.646, respectively) was slightly higher than that for MRI (0.725 and 0.598, respectively) without statistical significance ($P = 0.940$ and 0.716 ,

respectively). The short axis diameter of the metastatic lymph nodes (mean = 9.2 mm) was significantly larger than that of non-metastatic ones (mean = 5.17 mm, $P < 0.05$). Metastatic nodes had more irregular margins (44.4%) and central necrosis (22.2%) than non-metastatic ones (9% and 0%, respectively), with statistical significance ($P < 0.05$).

CONCLUSION: The accuracy of CT scan for the characterization of paraaortic nodes is not different from that of MRI. A short axis-diameter (> 5.3 mm), irregular margin, and presence of central necrosis are the suggestive morphologic features of metastatic paraaortic nodes.

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Key words: Paraaortic lymph node; Pancreatico-biliary carcinoma; Computed tomography; Magnetic resonance imaging

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INTRODUCTION

Paraaortic lymph node metastasis in the patients with pancreatico-biliary carcinoma has been reported as a definite predictor of early recurrence and shorter survival term, despite differences between individual tumors^[1-3]. It is very difficult to preoperatively predict paraaortic node metastasis with imaging, palpation, or intraoperative sonography. Therefore it is recommended that sampling and pathologic confirmation of paraaortic nodes should be performed before starting radical operation. Many surgeons, including

those in our hospital, do paraaortic node dissection before radical surgery^[4-7]. Although lymphadenectomy followed by histologic examination of the lymph nodes is still the gold standard for determination of metastasis, this procedure is invasive and could cause many surgical complications^[8-11]. Therefore preoperative, noninvasive imaging diagnosis of paraaortic node metastasis is very important^[12].

Lymph node staging in the various carcinomas has been extensively discussed in the previous literature^[13-19]. Dorfman *et al*^[20] reported that the upper limits of the normal nodes in the upper abdomen are site-specific. Therefore, site-specific nodal evaluation is necessary not only due to different clinical importance but also due to different morphologic criteria for malignancy^[21]. To our knowledge, however, there have been no radiologic reports on preoperative imaging diagnosis with a focus on the paraaortic node.

The purpose of our study was to compare computed tomography (CT) and magnetic resonance (MR) for preoperative detecting paraaortic lymph node metastasis in the patients with pancreato-biliary carcinoma and to re-define the significant morphologic features of metastatic ones.

MATERIALS AND METHODS

Patient population

The protocol for this study was approved by the Institutional Review Board at our institution and informed consent for this retrospective study was not required. From February 2000 to June 2006, 70 patients (37 men, 33 women; mean age, 62.9 years) with pancreatic head cancer ($n = 22$), ampulla of vater cancer ($n = 16$), distal common bile duct cancer ($n = 24$), or gallbladder (GB) cancer ($n = 8$) underwent CT ($n = 63$) and/or MR ($n = 38$) imaging. The mean interval time between lymphadenectomy and imaging evaluation was 16.7 d after CT and 18.3 d after MRI. Paraaortic lymphadenectomy was performed in all of the patients before or during surgical resection operations. Histological examinations revealed metastatic paraaortic nodes in 23 patients and non-metastatic nodes in 47 patients. Both CT and MRI were performed in 31 patients with pancreatic head cancer ($n = 11$), distal common bile duct cancer ($n = 13$), ampulla of vater cancer ($n = 6$) or GB cancer ($n = 1$). Nine patients had metastatic paraaortic nodes and 22 patients had non-metastatic nodes.

Imaging acquisition

All CT scans were obtained with one of the following commercially available multidetector or single detector CT scanners (Somatom Sensation 64, Somatom Sensation 64, Somatom Plus 4; Siemens Medical Solutions, Erlangen, Germany; Lightspeed Plus or QX/i, GE Medical Systems, Milwaukee, Wisconsin). Each patient received 120-150 mL of iopromide (Ultravist 300 or Ultravist 370; Schering, Berlin, Germany) at a rate of 3 mL/s. CT scans were obtained during the arterial phase (using a 25-35-s delay), portal venous phase (using a 70-75-s delay), and equilibrium phase (using a 3-min delay) after IV administration with 3-5-mm section thickness and 3-5-mm reconstruction interval.

MRI examinations were performed using a 1.5-T

imaging system (Gyrosan Intera, Philips Medical Systems Best, Netherlands), equipped with commercially available phased-array coils (Synergy; Philips Medical Systems, Best, Netherlands). Four-hour fasting was recommended before the examinations. Antiperistaltic agents or oral contrast agents were not used. The MRI protocol consisted of a breath-hold axial T1-weighted dual fast-gradient-recalled-echo sequence [(TR/in-phase TE, 180/4.6 ms; out-of-phase TE = 2.3 ms; flip angle, 90°; field of view, 32-36 cm × 25-29 cm; matrix, 240 × 240; section thickness, 7 mm; slice spacing, 7.7 mm; one signal acquired; number of slices = 24)]; a single shot turbo spin echo (TR/TE, 452/80 and 160; field of view, 32-36 cm × 25-29 cm; matrix, 288 × 230; section thickness, 7 mm; slice spacing, 5 mm; scan slices were overlapped by 2 mm using an interleaved acquisition technique) with spectral fat suppression and respiratory triggering technique; and a breath-hold transverse 3D gradient echo sequence with fat saturation (TR/TE, 3.9/1.1 msec; flip angle, 25°; field of view, 32-36 cm × 25-36 cm; matrix, 320/224; section thickness, 3 mm).

Contrast-enhanced MRI was performed using a breath-hold 3D gradient echo sequence with fat saturation sequence, following an IV bolus of 0.1 mmol gadobenate dimeglumine (MultiHance, Bracco SpA, Milan, Italy) per kilogram of body weight followed by a saline flush of 30 mL. This sequence was repeated four times with data acquisition in the hepatic arterial, portal venous, and equilibrium phases. An automatic infusion system (Spectris MR injection system, Medrad Europe, Maastricht, Netherlands) operating at an injection rate of 2 mL/s was used. The actual pulse sequence was started manually when the fluoroscopic sequence revealed that the contrast material bolus had reached the abdominal aorta.

Image analysis

All of the imaging analysis was performed on a picture archiving and communication system (PACS) workstation (Centricity 1.0; GE Medical Systems). This retrospective study was composed of two parts. To compare the diagnostic accuracy of CT and MRI, two radiologists independently evaluated preoperative CT and MR images within a 3-wk interval in 31 patients, without knowledge of final pathologic diagnosis. They considered the following criteria as the primary findings for metastatic nodes: (1) short diameter > 9 mm; (2) long axis diameter > 13 mm; (3) presence of necrosis; (4) irregular margin. Reviewers graded the paraaortic lymph node on a five-point scale of diagnostic confidence: 1, no node; 2, definitely benign; 3, probably benign; 4, probably metastatic; and 5, definitely metastatic. Diagnostic accuracy was evaluated using receiver operating characteristic (ROC) curve analysis with a calculation of the area (Az) under the ROC curve. Degree of interobserver agreement was expressed by a Kappa value; a kappa value greater than 0.60 indicated excellent agreement, between 0.40 and 0.60 was good, and less than 0.40 was poor^[22].

Using the CT and MR images, we redefined the morphologic criteria of metastatic nodes by comparing them with non-metastatic nodes. Two radiologists evaluated the CT scan in consensus for 63 patients (18 metastatic paraaortic nodes and 45 non-metastatic ones)



Figure 1 Metastatic right paraaortic lymph node in a 63-year-old man with pancreatic head cancer. **A:** Contrast-enhanced CT shows an irregularly shaped lymph node (arrow) with a short axis dimension of 11.5 mm that was interpreted as a definitely metastatic lymph node; **B:** Axial T1-weighted MRI shows an irregularly shaped lymph node (arrow) with a short axis dimension of 8.5 mm that was interpreted as a definitely benign lymph node. Pathologic examination revealed that this lymph node was metastatic.

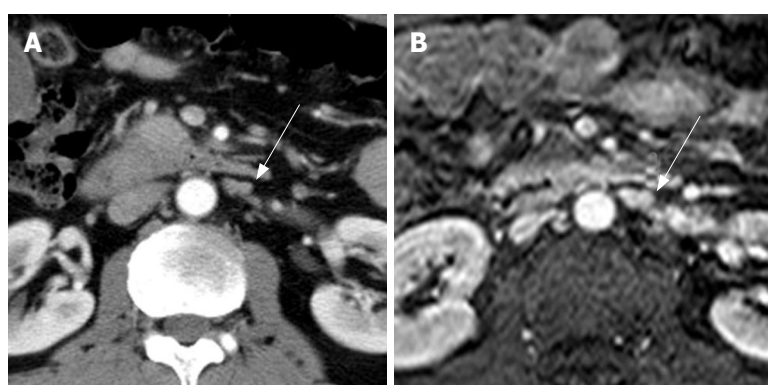


Figure 2 Metastatic left paraaortic lymph node in a 51-year-old man with pancreatic head cancer. **A:** Contrast-enhanced CT shows an irregularly shaped lymph node (arrow) with a short axis dimension of 7.2 mm that was interpreted as a probably metastatic lymph node; **B:** Axial contrast-enhanced T1-weighted MRI shows an irregularly shaped lymph node (arrow) with a short axis dimension of 7 mm that was interpreted as a probably metastatic lymph node; this diagnosis was confirmed by lymphadenectomy and pathological examination.

to record the short and long axial diameter and their ratio, margin (smooth or irregular), and the presence of necrosis in the detected paraaortic lymph nodes. The short and long axis diameter and their ratio were compared between metastatic paraaortic and non-metastatic lymph nodes by the independent samples *t*-test. The margin and presence of necrosis of metastatic paraaortic lymph nodes were compared to those of non-metastatic nodes by Fisher's exact test. $P < 0.05$ was considered statistically significant. A ROC curve was used to determine the best cut-off value for the short and long axis diameter for differentiation of metastatic from non-metastatic nodes. When multiple nodes in the paraaortic region were detected, the largest, irregular-shaped, and/or necrotic node was selected and defined as a metastatic node. The imaging findings were compared with histopathologic results on a per-case basis.

RESULTS

Accuracy of CT and MRI for detecting metastatic paraaortic lymph nodes

Interobserver agreement between the two readers for CT was excellent (kappa value 0.674; standard error 0.088), but that for MRI was poor (kappa value 0.359; standard error 0.157).

The mean area under the two readers' ROC curve for CT (0.732 and 0.646, respectively) was slightly higher than that for MRI (0.725 and 0.598, respectively) without statistical significance ($P = 0.940$ and 0.716 , respectively) (Figures 1 and 2).

Features of metastatic paraaortic lymph nodes on CT

The comparison between non-metastatic and metastatic

Table 1 Features of metastatic paraaortic lymph nodes on CT

	Non-metastatic	Metastatic	<i>P</i> value
Mean short diameter	5.17 mm	9.2 mm	< 0.05
Mean long diameter	8.72 mm	13.18 mm	< 0.05
Mean ratio (short/long)	0.58	0.7	0.284
Irregular margin	9%	44%	< 0.05
Necrosis	0%	28%	< 0.05

paraaortic lymph nodes on CT is summarized in Table 1. The short axis diameter of metastatic lymph nodes (mean = 9.2 mm, 3.8-28.1 mm) was significantly larger than that of non-metastatic lymph nodes (mean = 5.17 mm, 2.1-11.8 mm, $P < 0.05$). The long axis diameter of metastatic lymph nodes (mean = 13.18 mm, 5-32.1 mm) was significantly larger than that of non-metastatic lymph nodes (mean = 8.72 mm, 4.6-22.9 mm, $P < 0.05$). However, the ratio of the short to long axis of metastatic lymph nodes (mean = 0.70) was slightly larger than that of non-metastatic lymph nodes (mean = 0.58) without statistical significance ($P = 0.284$). The margins of the paraaortic lymph nodes were irregular in 8 of 18 patients (44.4%) with metastasis (Figures 1 and 2), and 4 of 45 patients (8.9%) without metastasis. The presence of central necrosis was seen in 4 of 18 patients (22.2%) with metastasis, but was not seen in patients without metastasis. Metastatic nodes had more irregular margins (44%) and central necrosis (28%) than non-metastatic nodes (9% and 0%, respectively), with statistical significance ($P < 0.05$).

Based on the ROC curve, we determined that the best cut-off values for differentiating metastatic nodes from non-metastatic nodes were > 5.3 mm for the short axis diameter



Figure 3 Two metastatic paraaortic lymph nodes in a 49-year-old man with gallbladder cancer. Axial (A) and coronal (B) contrast-enhanced CT shows several paraaortic lymph nodes. Among them, the right largest node (straight arrow) shows 10 mm and 18.8 mm of short and long axis diameters with irregular margin (on coronal image), compatible with metastatic node. The left one (dot arrow) shows 8.2 mm and 12.2 mm of short and long axis diameters, less than the mean value of metastatic ones (9.2 mm and 13.2 mm, respectively). According to the best cut-off value of short diameter more than 5.3 mm and long axis diameter more than 11.6 mm, The left one is also metastatic one rather than non-metastatic one. Pathologic examination revealed that two lymph nodes were metastatic ones among six resected paraaortic lymph nodes.

and > 11.6 mm for the long axis diameter (Figure 3). According to the short axis cutoff of > 5.3 mm, the diagnostic values for metastatic nodes were 77.8% sensitivity (95% confidence interval (CI): 52.4%-93.5%) and 66.4% specificity (95% CI: 48.8%-78.1%). According to the cutoff of > 11.6 mm for the long axis diameter, the diagnostic values for metastatic nodes were 50.0% sensitivity (95% CI: 26.1%-73.9%) and 91.1% specificity (95% CI: 78.8%-97.5%).

DISCUSSION

Although CT and MR imaging are well established for the staging and follow-up of patients with malignancy, the rates of accuracy for the detection of metastatic lymph nodes vary widely. It has been reported that the accuracy of CT and MRI for the detection of lymph nodes in patients with cervical carcinoma^[23-25] and the evaluation of regional nodes in the patients with rectal cancer^[26,27] is comparable. Other studies have suggested that CT is more specific for detecting positive lymph nodes in gynecologic cancers, whereas MR imaging is more sensitive^[23]. In some reports on the evaluation of cervical cancer, MRI (60%) was reported to be more sensitive than CT (43%), whereas the specificities of the two modalities were comparable^[28]. Focusing on paraaortic nodes in the patients with pancreato-biliary cancer, our study showed that the accuracy of CT and MRI were comparable. Our results revealed that the interobserver agreement for CT was excellent, whereas that for MR was poor. This finding suggests that the radiologist's experience is more important for evaluating by MRI than CT, although it is generally accepted that the tissue contrast with MRI is better than that with CT.

Size criteria have been used in the differentiation of metastatic from non-metastatic nodes, despite much dispute^[29]. In past, the maximum short axis diameter of a normal lymph node was known to vary on abdominal computed tomography, according to the node's location; the upper paraaortic region is 9 mm and the lower paraaortic region is 11 mm^[20]. A recent study for metastatic paraaortic nodes in pancreatic cancer shows that the size criteria combined with a long axis diameter (12, 10, 8, or 6 mm) and the axial ratio (0.5, 0.7, or 1.0) have a positive predictive value of 13% to 36% and an overall accuracy of 66.7% to 78.9%^[21]. Therefore it has been concluded that morphologic criteria are not useful in the evaluation of metastatic paraaortic nodes. A previous study of

gallbladder carcinoma, on the other hand, demonstrated a high positive predictive value (86%) in the evaluation of metastatic interaortocaval nodes based on the size and shape criteria; anterior posterior dimensions of 10 mm or larger and ring-like or heterogeneous contrast enhancement^[30]. In our study, there was a statistically significant difference between two groups: the mean values for the short and long axis diameter of metastatic paraaortic nodes were 9.2 mm and 13.18 mm, respectively, whereas those of nonmetastatic ones were 5.17 mm and 8.27 mm, respectively. In our study, the best cut-off value for differentiating metastatic nodes from non-metastatic nodes was a short axis diameter of more than 5.3 mm (77.8% sensitivity and 66.4% specificity) and a long axis diameter of more than 11.6 mm (50.0% sensitivity and 91.1% specificity).

It is well known that central necrosis has a very high positive predictive value (almost 100%) in the diagnosis of metastasis. Our study also demonstrated that central necrosis was seen only in metastatic nodes. However, central necrosis may be seen with tuberculosis. Moreover, the sensitivity of central necrosis is very low. In our study, irregular margin had a high positive predictive value, although it was not pathognomic.

Our study had some limitations. First, it was a retrospective study and the parameters of the CT and MRI were not uniform. Second, the imaging findings were compared with histopathologic results on a per-case basis not on a per-node basis.

In conclusion, we found that the accuracy of CT and MRI were comparable for the evaluation of paraaortic nodes in the patients with pancreato-biliary cancer. Central necrosis, irregular margin, and a cut-off value of more than 5.3 mm for the short axis diameter and 11.6 mm for the long axis diameter may be used as the criteria for diagnosing metastatic paraaortic nodes on CT scan. However, functional studies, such as high-resolution MRI with lymphotropic contrast agent, are necessary to overcome the limitation of morphologic evaluation of nodes.

COMMENTS

Background

In patients with pancreato-biliary carcinoma, paraaortic lymph node metastasis has a crucial impact on surgical indication or extent of operation. At present, many surgeons perform paraaortic lymphadenectomy for accurate assessment and

decision for adequate extent of operation. However, because of its invasiveness and complications, paraaortic lymphadenectomy for pancreatico-biliary carcinoma is controversial.

Research frontiers

Although a comparison between computed tomography (CT) and magnetic resonance (MR) has already been performed in cervical cancer, colorectal cancer and other malignancy, no studies to date have compared CT with MR in terms of detecting paraaortic lymph node metastases from pancreatico-biliary carcinoma. The aim of this study is to determine the accuracy of CT and MR for presurgical characterization of paraaortic lymph nodes in patients with pancreatico-biliary carcinoma.

Innovations and breakthroughs

The results of this study indicate that the accuracy of CT and MR were comparable for the evaluation of paraaortic nodes in the patients with pancreatico-biliary cancer. The lymph node diameter > 5.3 mm, irregular margin, and central necrosis are the suggestive morphologic features of metastatic paraaortic nodes.

Applications

CT and MR could be used for the selection of candidates for lymphadenectomy in the patients with pancreatico-biliary carcinoma.

Terminology

Paraaortic lymph node metastasis in the patients with pancreatico-biliary carcinoma has been reported as a definite predictor of early recurrence and shorter survival term.

Peer review

This is a very interesting paper, although it is a retrospective study. The idea of paraaortic lymph node in pancreatico-biliary is important for evaluation. This unique study will be a first step to confirm the results of a prospective study in the future.

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Thrombospondin-1 expression correlates with angiogenesis in experimental cirrhosis

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Abstract

AIM: To investigate the significance of Thrombospondin-1 (TSP-1) expression and its relationship with angiogenesis during experimental fibrosis.

METHODS: Cirrhosis was induced in male Wistar rats by intraperitoneal administration of diethyl nitrosamine (DEN). The serial sections from liver tissues were stained with anti-CD34 and anti-TSP-1 antibodies before being quantitated by light microscopy.

RESULTS: Our results showed that of TSP-1 expression gradually increases according to the severity of fibrosis (Group I vs group II, Group III and Group IV; Group II vs group III and group IV; group III vs group IV, $P < 0.05$). Moreover, TSP-1 expression was found to be correlated with angiogenesis ($P < 0.05$).

CONCLUSION: The correlative evidence of the link between TSP-1 and fibrosis or angiogenesis provided by this study suggests that besides its role as a strong promoter of transforming growth factor- β 1 (TGF- β 1), TSP-1 might have an additional role in liver fibrogenesis by stimulating angiogenesis and this protein could be a potential target to prevent fibrogenesis in chronic inflammatory diseases of the liver.

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Key words: Experimental liver cirrhosis; Immunohistochemistry; Liver fibrosis; Pathologic angiogenesis; Thrombospondin-1

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INTRODUCTION

Hepatic angiogenesis is frequently associated with inflammation and fibrogenesis during chronic liver injury^[1-5]. Currently, it is not clear whether this process plays a beneficial role in the maintenance of homeostasis or contributes to liver damage during chronic inflammation. However, the fact that chronic inflammatory liver diseases respond poorly to immunosuppressive and anti-inflammatory therapy suggests that angiogenesis might be a promising therapeutic target in the prevention of fibrosis^[6-8]. For this reason, attempts are being directed to evaluate the cellular and molecular mechanisms involved in the development of hepatic angiogenesis during chronic liver injury^[3-5].

Thrombospondin 1 (TSP-1), one of the five members of the Thrombospondin gene family, is a matrix protein involved in complex processes including wound healing and angiogenesis^[9,10]. The exact role of TSP-1 in angiogenesis is still controversial. TSP-1 can function as an inhibitor or as a promoter of angiogenesis, indicating that it might modulate this process in opposite directions^[11-16]. In malignant and premalignant conditions of the liver, TSP-1 expression and its association with angiogenesis have been demonstrated^[15-18]. Regarding non-neoplastic liver diseases, although the association of TSP-1 with latent transforming growth factor- β 1 (TGF- β 1) has been demonstrated in a few studies, the relationship between TSP-1 and angiogenesis during liver fibrogenesis has not been documented^[19-21].

Therefore, this study was undertaken to investigate the significance of TSP-1 expression during diethyl nitrosamine (DEN) induced experimental liver fibrosis and to evaluate whether any relationship exists between TSP-1 expression and angiogenesis.

MATERIALS AND METHODS

Materials

This animal study was approved by the local animal ethics committee of the Akdeniz University. Male adult Wistar rats weighing 250 g were used. They were maintained on

Table 1 Distribution of mean, standard deviation, median and ranges of VD and TSP-1 expression in normal livers (group I) and in DEN treated livers (group II, group III, and group IV)

Group	VD ^a			TSP-1 expression (%) ^a		
	mean \pm SD	Median	Ranges	mean \pm SD	Median	Ranges
I (n: 8)	3.24 \pm 1.41	3	2-6	1.63 \pm 1.06	1.5	0-3
II (n: 9)	5.22 \pm 1.86	5	2-8	5.89 \pm 1.18	4	0-14
III (n: 10)	9 \pm 4.57	6	1-16	16.3 \pm 7.32	9	2-26
IV (n: 10)	14.5 \pm 5.97	11	8-26	68.5 \pm 19.73	44	0-95

n: Number of cases; VD: Vascular density; SD: Standard deviation; ^a $P < 0.05$.

a commercial diet and water in a room at $22 \pm 2^\circ\text{C}$ under normal laboratory lighting conditions.

Methods

Animal model: The rats received intra-peritoneal injections of DEN (Sigma, Saint Quentin Fallavier, France) at 100 mg/kg of body weight (n: 29) or 0.9% sodium chloride (n: 8) once a week. The injections were performed for 2 (n: 4), 4 (n: 5), 5 (n: 5), 6 (n: 5), 8 (n: 5) and 10 (n: 5) wk. The animals were sacrificed 2 wk after the last administrations and a hepatectomy was performed. Liver tissue samples were either frozen immediately in liquid nitrogen and stored at -70°C or fixed in 10% buffered formalin and embedded in paraffin.

Histology and immunohistochemistry: Four micrometer thick serial sections from the liver tissues originally fixed in formalin and embedded in paraffin were prepared and stained with hematoxylin and eosin for the histopathological assessment. Masson trichrome staining was used in the evaluation of the extent of liver fibrosis.

Immunolabeling was performed using polyclonal antibodies directed anti rat CD34 (sc-7045 goat, dilution: 1:500, Santa Cruz, CA, USA) and thrombospondin-1 (sc-12312 goat, IgG, dilution 1:200, Santa Cruz, CA, USA). An avidin-biotin-peroxidase technique (sc-2023, anti-goat ABC staining Kit; Santa Cruz, CA, USA) was used for labeling. For CD34, sections from paraffin embedded tissue blocks were dried in a hot air oven at 55°C overnight and dewaxed. Microwave antigen retrieval (750 W, 4×5 min in citrate buffer 0.01 mol/L, pH 6) was performed. TSP-1 staining was applied to 5 μm thick air dried (30 min) cryostat sections, fixed in acetone (10 min). Endogenous peroxidase was blocked by using 3% hydrogen peroxide in methanol for 30 min. Each step of incubation was followed thorough washing of the slides in phosphate buffered saline (PBS). After incubation with primary antibody against CD34 and TSP-1 (each 30 min), sections were reacted with secondary biotinylated antibody (30 min) and AB enzyme reagent (avidin and biotinylated horseradish peroxidase) for 30 min. Finally, all slides were treated with DAB reagent to develop color and counterstained with Mayer's hematoxylin. Normal goat and rabbit IgG instead of primary antibodies were used as negative control at the same dilution.

The vascular density in portal and periportal areas was assessed by determining the count of CD34 labeled vessel sections at higher magnification ($\times 400$) with the use of

an ocular grid subdivided into 100 areas. For each subject vascular density (VD) was noted.

For quantitative evaluation of TSP-1 expression, in each section positive and negative cells were counted in systematically randomly selected 10 to 15 microscopic fields by using an ocular grid at high magnification ($\times 400$). The positive staining was calculated as the percentage of positive cells to total number of counted cells. Positive cells touching the left and lower edge of the grid were not included.

All analysis were performed using Statistical Package for Social Science (SPSS 15.0 for Windows, USA). Mann-Whitney-U test was used to establish the difference between groups. Friedman test was used to determine the relationship between quantitative parameters. Data were expressed as mean \pm SD and $P < 0.05$ was considered significant.

RESULTS

In this study, fibrogenesis was not observed in the control group. In DEN treated rats, fibrous septa were detected after 5 wk. The liver was cirrhotic in all cases after 8 wk. According to the severity of fibrosis, cases were divided in following groups: Group I: normal livers, group II: non-fibrotic livers (2 and 4 wk), group III: fibrotic livers (5 and 6 wk) and group IV: cirrhotic livers (8 and 10 wk) (Figure 1A). In group I, CD34 staining was restricted to the endothelium of portal vessels. While in non-fibrotic livers CD34 expression was noted in a few vascular structures around portal areas, numerous CD34-labeled vessels were detected in fibrotic livers. In group IV, CD34 staining revealed a dense vascular plexus surrounding the cirrhotic nodules (Figure 1B). Parallel to this finding, VD values were increased together with the progression of fibrosis (Figure 2). DEN-treated cases (group II, III and IV) had higher VD than the control group ($P < 0.05$). The difference between VD values of group II, III and IV was also statistically significant ($P < 0.05$) (Figure 2 and Table 1).

In normal livers (group I), TSP-1 expression was restricted to the endothelium of portal vessels and to a few hepatocytes (Figure 1C). However, in non-fibrotic group TSP-1 expression was higher than normal livers with more positive hepatocytes and perisinusoidal cells ($P < 0.05$). TSP-1 expression continued to increase in fibrotic livers and was more widespread in cirrhotic livers. The expression of TSP-1 in DEN-treated rat groups was significantly different from each other ($P < 0.05$) (Figure 1C, Figure 2 and Table 1).

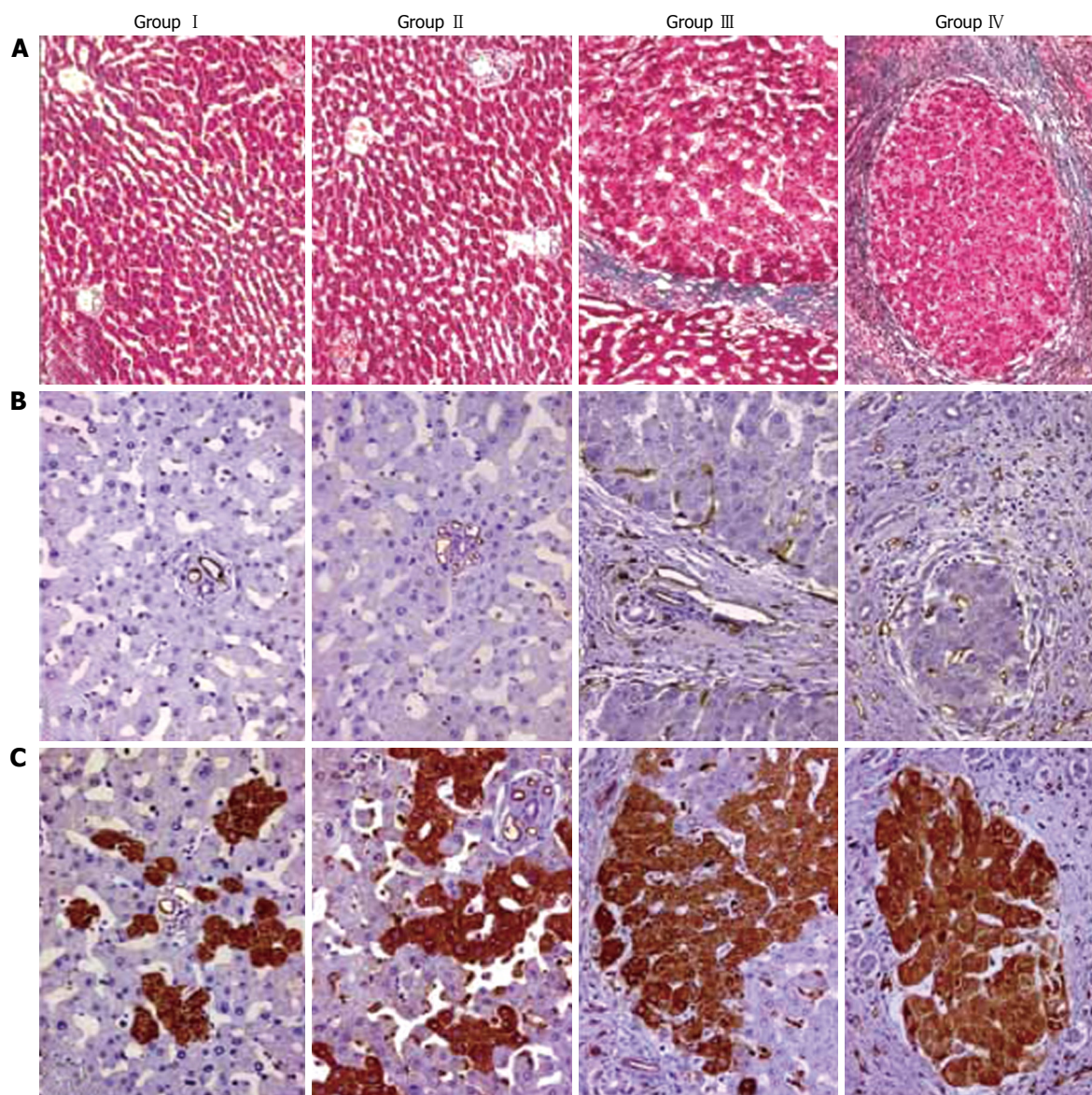


Figure 1 Liver fibrosis (A), angiogenesis (B) and TSP-1 expression (C) in the study group. Liver fibrosis was stained by Masson trichrome at different time points of treatment and angiogenesis was evaluated with an anti-CD34 antibody. In normal livers, the number CD34 labeled vessels and TSP-1 positive cells is lower when compared to DEN treated livers. In the latter, their number increases according to the extent of fibrosis.

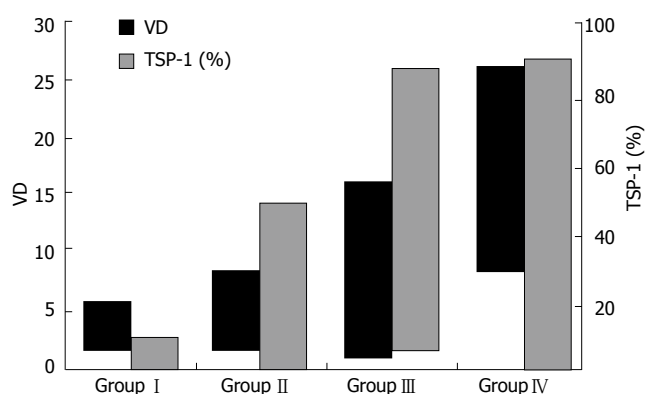


Figure 2 Results of the quantitative assessment of angiogenesis and TSP-1 expression in normal and DEN treated rat livers. There is a gradual increase for VD and TSP-1 expressions parallel to the severity of fibrosis.

Friedman test showed that there was a significant correlation between VD and TSP-1 expression ($P < 0.05$).

DISCUSSION

Results of the recent studies emphasized that hepatic angiogenesis is associated with fibrogenesis in the wound healing response to chronic liver injury^[11-13]. In our study, parallel to this finding, angiogenesis, assessed as VD, was increased with the progression of fibrosis ($P < 0.05$). Besides, in group II, despite the absence of overt fibrosis, VD was higher than that of normal livers, suggesting that angiogenesis is an early event which might take place before the onset of fibrosis during chronic liver damage.

It is well known that angiogenesis does not involve a single pathway, but is a complex event regulated by many angiogenic and antiangiogenic factors, including TSP-1^[9,10]. In neoplastic and premalignant conditions of the liver, the relationship between TSP-1 expression and angiogenesis has been studied^[15-17]. However, in non-neoplastic liver diseases the association of TSP-1 expression with angiogenesis and its role in this complex event has not

been documented. Because TSP-1 is also a known activator of TGF- β 1, a key mediator in tissue fibrogenesis, a few studies has been focused to evaluate the effect of TSP-1 in hepatic activation of TGF- β 1^[19-21]. It was concluded that TSP-1 may act in the pathogenesis of liver fibrogenesis as a strong promoter of TGF- β 1. Although in the present study TGF- β 1 expression was not evaluated, we observed an increase of TSP-1 expression parallel to the severity of fibrosis. TSP-1 expression of normal livers was restricted to the endothelium of portal vessels and to a few hepatocytes. However, this value was 3.61 fold higher in non-fibrotic group. The percentage of TSP-1 expressing cells continued to increase in fibrotic and cirrhotic livers ($P < 0.05$). The present data support the contribution of TSP-1 expression in the wound healing response to chronic liver injury^[19-21]. Moreover, in this study, a strong correlation between TSP-1 expression and angiogenesis was observed ($P < 0.05$). This finding suggests that TSP-1 is not only involved in fibrogenesis by the hepatic activation of TGF- β 1 but also might play another role in the remodeling of the liver architecture by contributing to the development of angiogenesis.

Our results showed TSP-1 might be a stimulator of angiogenesis during liver injury. TSP-1 is generally recognized as an antiangiogenic agent^[11,12,15]. However results of the some studies have been demonstrated that TSP-1 might be a stimulator of angiogenesis^[13,14,16]. For this reason the actual role played by TSP-1 in angiogenesis has been investigated in previous studies with several different conclusions. Some studies demonstrated that the effect of TSP-1 may depend on its concentration^[13,22], the type of domain being activated^[23] and the type of receptors present on endothelial cells^[24]. It has been also speculated that the actual role of TSP-1 might be related to number of its receptors^[25]. Although it is not possible to conclude, based on our findings, which factor determines the angiogenic effect of TSP-1 during chronic liver injury, our data reinforce its dual role in the modulation of angiogenesis in opposite directions.

In conclusion, the results of this descriptive study reveal that in experimental liver fibrogenesis TSP-1 expression gradually increases according to the severity of fibrosis and strongly correlates with angiogenesis. Our data suggest that TSP-1 might contribute to the wound healing response to liver injury not only as a strong promoter of TGF- β 1, but also as an inducer of angiogenesis. In the light of this observation, it would be of interest to evaluate the mechanism triggered by TSP-1 in hepatic angiogenesis with further experimental models, in order to completely clarify if TSP-1 could be a potential target in the manipulation of angiogenesis in chronic inflammatory liver diseases ending with cirrhosis.

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COMMENTS

Background

Angiogenesis progresses together with fibrogenesis in the wound healing

response to chronic liver injury. Thrombospondin-1 (TSP-1) is a matricellular protein which is involved in complex processes including wound healing and angiogenesis. TSP-1 might modulate angiogenesis in opposite directions. In malignant and premalignant conditions of the liver the relationship between TSP-1 expression and angiogenesis have been demonstrated. Regarding non-neoplastic liver diseases, although the association of TSP-1 with latent transforming growth factor- β 1 (TGF- β 1) has been demonstrated in a few studies, the relationship between TSP-1 and angiogenesis during liver fibrogenesis has not been documented.

Research frontiers

At present it is not possible to ascertain the exact pathogenic role of angiogenesis in liver fibrogenesis. However, the fact that chronic liver diseases respond poorly to conventional therapies suggests that manipulation of angiogenesis could be a promising approach to treatment. For this reason, the cellular and molecular mechanisms that are involved in the development of angiogenesis during liver fibrogenesis have been a topic of intensive investigations in the recent years.

Innovations and breakthroughs

This study demonstrated that in experimental liver fibrogenesis TSP-1 expression gradually increases according to the severity of fibrosis and strongly correlates with angiogenesis.

Applications

Based on the results of this research, TSP-1 might contribute to the wound healing response to liver injury not only as a strong promoter of TGF- β 1, but also as an inducer of angiogenesis and could be a potential target in the manipulation of angiogenesis in chronic inflammatory liver diseases ending with cirrhosis.

Terminology

TSP-1 is a high molecular weight glycoprotein (450 kDa) which is composed of three identical subunits cross-linked by disulfide bonds. Each subunit is composed of several domains interacting with different surface receptors. TSP-1 is involved in various processes such as cell motility, inflammation and wound healing. It also modulates endothelial cell adhesion, motility and growth.

Peer review

This is quite an interesting investigational paper. This study demonstrated that in experimental liver fibrogenesis, TSP-1 expression gradually increases according to the severity of fibrosis and strongly correlates with angiogenesis. Further study would focus on evaluating the mechanism of TSP-1 in hepatic angiogenesis.

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RAPID COMMUNICATION

Sustained virological response based on rapid virological response in genotype-3 chronic hepatitis C treated with standard interferon in the Pakistani population

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Abstract

AIM: To document the sustained virological response (SVR) in rapid virological responders (RVR) of genotype-3 chronic hepatitis C with standard interferon (SdIF).

METHODS: Hepatitis C genotype-3 patients during the period July 2006 and June 2007 were included. Complete blood counts, prothrombin time, ALT, albumin, qualitative HCV RNA were done. SdIF and ribavirin were given for 4 wk and qualitative HCV RNA was repeated. Those testing negative were allocated to group-A while the rest were allocated to group-B. Treatment was continued a total of 16 and 24 wk for group A and B respectively. HCV RNA was repeated after 24 wk of treatment. End virological and sustained virological responses were compared by χ^2 test. ROC of pretreatment age, ALT and albumin were plotted for failure to achieve SVR.

RESULTS: Of 74 patients treated, RCV RNA after 16 wk of therapy became undetectable in 34 (45.9%) and was detectable in 40 (54.1%) and were allocated to groups A and B respectively. SVR was achieved in 58.8% and 27.8% in groups A and B respectively. SVR rates were significantly higher in patients who had RVR as compared to those who did not ($P = 0.0$; $\gamma = 2$). Both groups combined ETR and SVR were 70% and 33% respectively. ROC plots of pretreatment age, ALT and albumin for SVR showed only ALT to have a significantly large area under the curve.

CONCLUSION: SVR rates were higher in patients who had RVR with SdIF and high pre treatment ALT values correlated to probability of having RVR.

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Key words: Hepatitis C; Sustained virological response; Rapid virological responders; Chronic hepatitis

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Zuberi BF, Zuberi FF, Memon SA, Qureshi MH, Ali SZ, Afsar S. Sustained virological response based on rapid virological response in genotype-3 chronic hepatitis C treated with standard interferon in the Pakistani population. *World J Gastroenterol* 2008; 14(14): 2218-2221 Available from: URL: <http://www.wjgnet.com/1007-9327/14/2218.asp> DOI: <http://dx.doi.org/10.3748/wjg.14.2218>

INTRODUCTION

Hepatitis C virus (HCV) is a big health care problem all over the world with 130 million patients infected with this virus world over^[1,2]. The problem in developing countries is compounded by the poor economical status of the patients who are unable to afford the expensive therapy. The most prevalent genotype in Pakistan is type-3 (68%-87%) which has a favorable response to standard interferon^[3-6]. Recently some studies have suggested 16 wk therapy with pegylated interferon (PgIF) in patients who achieve rapid virological response (RVR) at 4 wk. The SVR rates in patients with RVR with pegylated interferon have been reported at 78% and that with standard interferon (SdIF) at 53%^[7]. The treatment protocols that consider viral load and RVR are proven to be more cost effective than standard protocols^[8]. For genotype-2 most of the studies are in favour of a 16 wk therapy in patients who achieve RVR but there is no such recommendation for genotype-3 and more data is required to make any such recommendation^[9].

In a poor country like Pakistan affordability of even 16 wk of PgIF treatment is financially difficult. There is

no report of 16 wk standard interferon therapy results regarding sustained virological response (SVR) from Pakistan. The current study was conducted to see the end treatment response (ETR) and SVR in patients who achieved the RVR with SdIF.

MATERIALS AND METHODS

Subjects

This interventional study was conducted at Civil Hospital and Anklesaria Nursing Home Karachi during the period July 2006 and June 2007. Naïve patients of chronic HCV of Genotype-3 were included. Patients with decompensated disease, depression, allergy to interferon were excluded. Complete blood counts (CBC), prothrombin time (PT), ALT, albumin, qualitative HCV RNA and genotype were done before start of therapy. Therapy was started with SdIF α 2a 3.0 MU thrice weekly (TIW) and ribavirin 800-1200 mg PO according to weight. Qualitative HCV RNA was repeated after 4 wk into therapy. Patients who tested negative at this stage were allocated to Group-A while those in which it was still detectable were allocated to Group-B. Patients in Group-A were continued with the same therapy for 12 more wk for a total of 16 wk and then the therapy was stopped. These patients were retested for HCV RNA after 24 wk of stoppage of therapy for SVR. Patients in Group-B were continued with the same therapy for a total of 24 wk and were retested for HCV RNA for ETR. Patients in which HCV RNA was still detected after 24 wk of therapy were labeled as non-responders. Those who tested negative were followed for a further 24 wk without any treatment and retested for HCV RNA for SVR (Figure 1).

Methods

CBC was done by a Sysmex Autoanalyzer while biochemical tests were done by Hitachi Autoanalyzer using Merck biomedical reagents. HCV RNA was done by Roche reverse transcriptase method. Sample size was determined for hypothesis testing for two population proportions^[10]. Keeping the level of significance at 5%, power of study at 90% and reported SVR rates of 78% and 53% the sample size was calculated to be of 74 patients^[7]. Scale variables of age, ALT, hemoglobin, albumin were compared between the two groups by Students *t*-test. The nominal variables of gender, ETR & SVR were compared by χ^2 test. Receiver operator curve (ROC) was plotted for age, ALT and albumin at the start of treatment. Failure to achieve RVR was taken as a variable state.

Statistical analysis

The significant level was set at ≤ 0.05 . SPSS version 15.0 was used^[11].

RESULTS

Seventy four eligible patients of genotype-3 HCV satisfying the selection criteria were included. These included 50 (67.6%) males and 24 (32.4%) females. Mean age of males was 35.9 ± 8.0 years and that of females was 39.1 ± 8.1 years. No statistically significant difference was found

Table 1 Demographic details of studied population

	Group A (n = 34)	Group B (n = 40)	γ	P	95% CI
Age (yr)	35.7 \pm 8.2	38.0 \pm 8.0	72	0.110	-7.198 to 0.751
M:F	22:12	28:12	1	0.804	-
ALT (U/L)	117.5 \pm 38.4	160.4 \pm 38.6	72	0.000	-60.770 to -24.971
Albumin (g/L)	38 \pm 7	38 \pm 4	72	0.789	-0.312 to 0.238

between the ages of the two genders ($P = 0.11$; $\gamma = 72$; 95% CI = -7.7 to 0.8) (Table 1). Patients were started with SdIF 3.0 MU TIW SQ with ribavirin according to body mass. RVR was achieved in 34 (45.9%) of the patients. These patients were allocated to Group-A while the rest of 40 (54.1%) patients who didn't achieve RVR were allocated to Group-B. Patients in Group-A were continued with the same treatment for a further 12 wk making a total of 16 wk of therapy. They were followed off treatment for a period of 24 wk and HCV RNA was repeated for SVR. Among these 20 (58.8%) patients achieved the SVR while 14 (41.2%) had a relapse within 24 wk of the follow-up period. In Group-B after 24 wk of therapy the ETR was achieved in 18 patients while 22 patients did not respond and were excluded from further analysis. After the off treatment follow-up of 24 wk in patients who achieved the ETR in group-B, the SVR was present in 5 (27.8%) and relapse was detected in 13 (72.2%) patients. Comparing the SVR rates between the two groups, SVR rates were statistically higher in Group-A ($P = 0.044$; $\gamma = 1$). SVR rates were significantly higher in patients who had RVR as compared to those who didn't; 20/34 (58.8%) *vs* 5/40 (12.5%) ($P = 0.0$; $\gamma = 2$). The combined outcome results show that RVR was achieved in 34 (45.9%), ETR in 52 (70.3%) & SVR in 25 (33.8%). The total relapse rate was 27 (36.5%) while the total non-responder rate was 22 (29.7%).

ROC plots for age, ALT, and albumin at the time of induction were plotted for failure to achieve RVR at the end of 4 wk (Figure 2). The area under the curve for ALT was significantly high at 0.8 with $P = 0.000$. This shows that among the three variables ALT had strong predictive value for RVR failure. At the cutoff of 73.5 IU/dL the sensitivity and specificity of ALT for RVR failure was 1.0 and 0.94 respectively. Thus values of ALT ≤ 73.5 U/L at the start of treatment were less likely to have RVR.

DISCUSSION

Although much research has gone into the treatment of chronic HCV, the optimal treatment is not yet established^[12]. Many treatment options are in vogue with different success rates^[13-15]. PgIF based protocols have better responses as compared to the SdIF but cost becomes the major hurdle in developing countries. In Pakistan the most common genotype is 3 which is highly responsive to SdIF^[15,16]. RVR is now a new landmark in the treatment and retreatment of HCV and it not only determines the duration but also predicts the outcome of the therapy^[17-19]. For genotype-2 most of the studies are in favour of a 16 wk therapy in patients who achieve RVR but there is no such recommendation for genotype-3^[9].

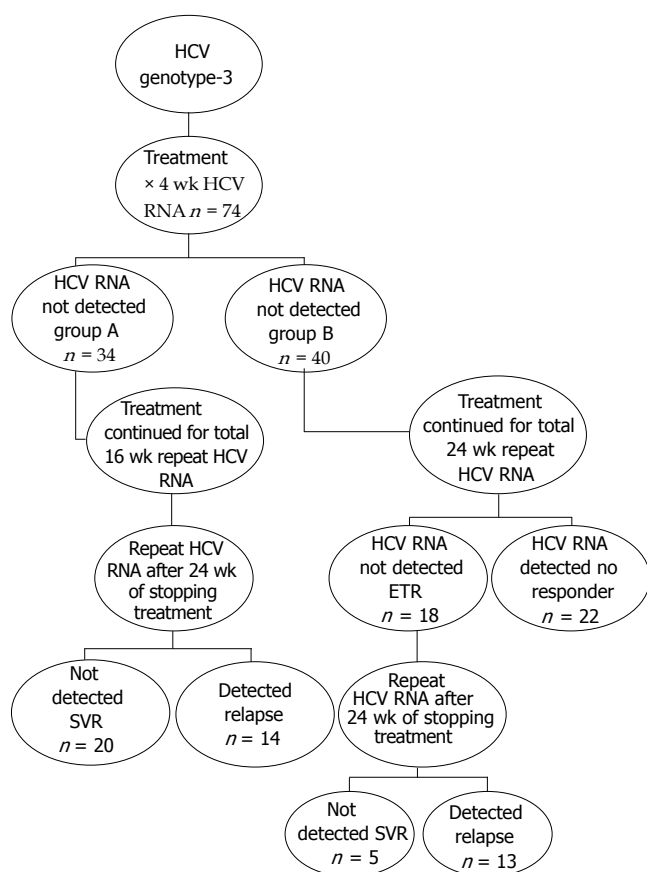
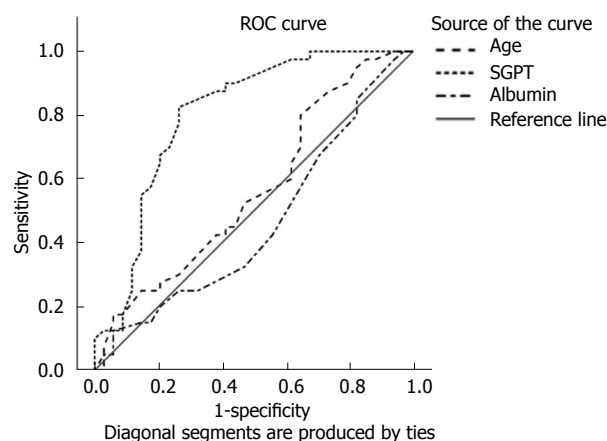


Figure 1 Treatment algorithm flowchart.

Most of the studies with RVR were done in western countries which have a high prevalence of genotype-1. We report our results with SdIF in genotype-3 HCV; with about 46% achieving RVR and about 59% among them achieving SVR. A recent study by Yu *et al*^[20] with PgIF reported RVR and SVR at 86% and 94% respectively. Another recent study by Yu *et al* reported RVR with PgIF in genotype-3 at 60%^[17]. Cost effectiveness of the RVR based therapy with PgIF is established by recent reports not only in HCV infection alone but also in combined HCV/HIV infections^[7,8]. In Pakistan the majority of genotype-3 patients are treated with SdIF due to economic reasons and Pakistan Society of Gastroenterology and GI Endoscopy also favours the use of SdIF in genotype-3^[21]. Government is also providing only SdIF *via* a special Prime Minister's initiative for a viral hepatitis program, thus PgIF is out of reach for the majority of the patients.

The results of our study with SdIF are not comparable to that with PgIF in genotype-3 HCV on both RVR and SVR. In our study the SVR rates in patients who achieved RVR were about 59% and over all both groups combined SVR was 33% only which are quite low as compared to the recent reports of 86% and 90%^[20]. Our study did show that SVR rates were higher in patients who attained RVR showing that RVR is an important landmark in management of HCV with SdIF too.

No data is available for prediction of RVR in patients undergoing treatment for HCV, although some data is available for SVR. One such study has been reported



Area under the curve					
Test result variable (s)	Area	Std. Error (a)	Asymptotic Sig. (b)	Asymptotic 95% CI	
				Upper bound	Lower bound
Age	0.566	0.068	0.332	0.433	0.698
ALT	0.802	0.054	0.000	0.696	0.909
Albumin	0.460	0.068	0.554	0.326	0.594

Figure 2 ROC Curve for age, ALT and Albumin for relapse.

that at the age of 20 years; no cirrhosis/bridging fibrosis; ALT quotient = 7; body mass index 20 kg/m²; viral load 40×10^6 IU/L was associated with a 97% probability of SVR^[22].

In this study we tested the pretreatment levels of ALT, albumin and age as a prediction for RVR by plotting the ROC plots. Only ALT was found a significant marker as patients with high ALT were more likely to achieve RVR.

In conclusion, SVR rates were higher in patients who had RVR with SdIF and high pre treatment ALT values correlated to probability of having RVR.

COMMENTS

Background

Treatment of Hepatitis C virus (HCV) has varied response according to the genotype of the infecting virus. Ability to achieve Sustained Virological Response (SVR), which is the HCV polymerase chain reaction (PCR) negativity maintained six months after stopping treatment, is the real objective of the treatment. It is difficult to predict who will achieve Rapid virological response (SVR).

Research frontiers

Introduction of SVR is the clearance of HCV RNA within one month of treatment. It is being projected as a marker for SVR in patients treated with pegylated interferon. RVR has not been studied for SVR with standard interferon.

Innovations and breakthroughs

The study documents that in HCV genotype-3 patients treated with standard interferon SVR is related to the RVR. It was also shown that patients with initial high ALT are more likely to have RVR.

Applications

This will allow for better prediction of treatment results.

Terminology

SVR: Sustained virological response is defined as HCV RNA negativity six months after stopping the treatment. RVR: Rapid virological response is HCV RNA negativity after one month of treatment.

Peer review

Authors have shown that a short course of short acting interferon (16 wk) is efficacious in the patients who experience EVR. Perhaps, this approach would be cost-effective and useful in Pakistan.

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RAPID COMMUNICATION

Cost saving by reloading the multiband ligator in endoscopic esophageal variceal ligation: A proposal for developing countries

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ligator thus is recommended especially for developing countries where most of the patients are not health insured.

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Key words: Esophageal varices; Reloading; Multiband ligator; Eradication; Cost saving

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Abstract

AIM: To assess the cost savings of reloading the multiband ligator in endoscopic esophageal variceal ligation (EVL) used on the same patient for subsequent sessions.

METHODS: This single centre retrospective descriptive study analysed patients undergoing variceal ligation at a tertiary care centre between 1st January, 2003 and 30th June, 2006. The multiband ligator was reloaded with six hemorrhoidal bands using hemorrhoidal ligator for the second and subsequent sessions. Analysis of cost saving was done for the number of follow-up sessions for the variceal eradication.

RESULTS: A total of 261 patients underwent at least one session of endoscopic esophageal variceal ligation between January 2003 and June 2006. Out of 261, 108 patients (males 67) agreed to follow the eradication program and underwent repeated sessions. A total of 304 sessions was performed with 2.81 sessions per patient on average. Thirty-two patients could not complete the programme. In 76 patients (70%), variceal obliteration was achieved. The ratio of the costs for the session with reloaded ligator *versus* a session with a new ligator was 1:2.37. Among the patients who completed esophageal varices eradication, cost saving with reloaded ligator was 58%.

CONCLUSION: EVL using reloaded multiband ligators for the follow-up sessions on patients undergoing variceal eradication is a cost saving procedure. Reloading the

Abbas Z, Rizvi L, Ahmed US, Mumtaz K, Jafri W. Cost saving by reloading the multiband ligator in endoscopic esophageal variceal ligation: A proposal for developing countries. *World J Gastroenterol* 2008; 14(14): 2222-2225 Available from: URL: <http://www.wjg-net.com/1007-9327/14/2222.asp> DOI: <http://dx.doi.org/10.3748/wjg.14.2222>

INTRODUCTION

Variceal hemorrhage is a major cause of death among patients with cirrhosis, carrying historically, a mortality rate of up to 50% before the advances in medicine^[1,2]. Even with the advent of intensive care, vasoactive medications, and endoscopic therapies, the risk of death with variceal hemorrhage is still about 20% per episode^[3,4].

Band ligation of esophageal varices is indicated as a primary prophylaxis for large varices and as a secondary prophylaxis for patients who have bled from varices^[5]. It is the endoscopic procedure of choice to prevent recurrent variceal hemorrhage and eradicate varices which usually requires 3-4 sessions^[6,7]. Endoscopic variceal ligation (EVL) is an expensive procedure, especially for patients from lower socioeconomic class in developing countries where health insurance and reimbursement systems are not as developed as in other countries. Most of the expenses are due to the high costs of the single polyband ligator use. Thus, reloading this ligator and re-using it for subsequent sessions on the same patient would substantially reduce costs, while also improving compliance to the eradication program as most of the patients are not covered by a health care scheme.

The aim of this study is to review the patients in the eradication program for esophageal varices and estimate the cost saving by using the reloaded band ligator to achieve this purpose.

MATERIALS AND METHODS

A retrospective analysis on 261 patients who had undergone EVL as primary or secondary prophylaxis between 1st January 2003 and 30th June 2006 was performed.

Saeed's Six Shooter Multi-Band Ligator (Cook Medical Inc, Bloomington, IN) was used for variceal ligation^[8]. After each session all the accessories of the ligator were disinfected in glutaraldehyde solution (Cidex, Johnson & Johnson) by standard protocols. The band ligator was then reloaded with six hemorrhoidal bands for the 2nd and subsequent sessions on the same patient. We used hemorrhoidal band ligator for reloading barrel of the variceal ligator^[9]. The procedure was approved by the Infection Control Committee of the hospital.

The procedures were performed by physicians experienced in the techniques of endoscopic ligation and sclerotherapy. Informed consent was obtained from the patients. Endoscopy was carried out under topical or pharyngeal anesthesia and sedation with intravenous midazolam if needed. Ligation was performed beginning at the most distal discernible extent of a variceal column and proceeding proximally. Subsequent endoscopic therapy sessions with EVL or combination therapy were performed at 14 to 21 d intervals until the varices were eradicated or reduced to grade one. Recurrent bleeding mandated unscheduled intervention.

Method for reloading

The plaited string or trigger cord of the multiple band ligator becomes separated into two threads near the barrel. Each thread has six beads at regular intervals starting from the tip of the thread. These threads are passed through the barrel of the multiple band ligator from its scope-end side and delivered from the transparent rim side. The banding apparatus is now loaded. The metal cone of the hemorrhoidal ligator is loaded with a band, and then fitted in to the cylinder of the hemorrhoidal ligator and the rubber band rolled from the cone to the cylinder. The cone is removed after charging the cylinder. The first tip (bead) of each thread is brought at the base of the transparent cap and held in position. The transparent rim of the barrel is slid into the cylinder and the handle of the hemorrhoidal ligator is closed to push off the band from the cylinder onto the barrel of the variceal ligator. The band is positioned to the base of the barrel's transparent portion above the first pair of beads. The next pair of beads is now brought above the first band, wrapping the portion of thread between the first and second bead on the barrel by repositioning second beads to 180 degrees. When the two beads are in position above the first band, the second band is applied. In this way all the bands are mounted on the barrel which is now ready for reuse.

RESULTS

A total of 261 patients underwent at least one session

Table 1 Cost savings after the first session in 76 patients who completed the eradication of varices. Cost is in US Dollars (1 US Dollar = 61 Pakistani Rupees)

	Reloaded band after first session	New six shooter used each time
Cost of EGD	91.8	91.8
Cost of bands/ligator	6.56	140.82
Cost of single follow up session	98.36	232.62
Cost of bands in 139 follow up session	911.47	19573.93
Total cost of 139 follow-up sessions	13672.13	32334.59
Average cost savings per patient	245.56	
Cost comparison	1	2.37
Overall cost saving	58%	
Cost saving in band ligators	95%	

Cost of EGD includes both the costs of the technical (i.e. equipment and facility costs) and professional fees.

of EVL between January 2003 and June 2006. Patients undergoing sclerotherapy were not included in the study.

Out of 261, 108 patients agreed to follow the eradication program with reloaded band ligator and underwent a total of 304 sessions. Sixty-seven (62%) patients were males. They underwent 2.81 sessions on average. Twenty patients came only for one follow up session, while 12 patients underwent more than one follow-up session but did not complete esophageal varices eradication. Thus, a total of 76 (70%) patients participating in the program achieved eradication. These 76 patients completed esophageal varices eradication in 215 sessions (average 2.83). The reloaded ligator was used in a total of 139 follow-up sessions. The ratio of costs for the session with the reloaded ligator *versus* a first session with a new ligator was 1:2.37. Among the patients who completed the program and achieved eradication of esophageal varices, cost saving with reloading was 58% (Table 1).

The etiologies of esophageal varices among the patients in the eradication program included hepatitis C in 49 (64.5%), hepatitis B in 3 patients (3.9%), hepatitis B & D in 6 (7.9%), non-B non-C in 16 (21.1%), and alcoholic liver disease in 2 patients (2.6%).

DISCUSSION

Cirrhosis and complications of portal hypertension rank among the top 10 leading causes of death worldwide^[10]. The prevalence of esophageal varices in patients with cirrhosis ranges from 12% to 90% and the average risk of bleeding from 14% to 78%, depending on the patient population studied^[11]. Esophageal varices are the most common cause of significant gastrointestinal bleeding secondary to portal hypertension^[12]. The acute mortality of variceal hemorrhage has been reported to be 15%-50% and the overall mortality within 1-4 years as high as 70%-80% in those with cirrhosis. Furthermore, once varices have bled, the risk of rebleeding is reported to be as high as 70%-80%.

Treatment of patients with esophageal varices includes the prevention of the initial bleeding episode (primary prophylaxis), the control of active hemorrhage, and the prevention of recurrent bleeding after a first episode

(secondary prophylaxis), for which several modalities have been used including endoscopic sclerotherapy and band ligation.

EVL is superior to sclerotherapy, and is considered to be the endoscopic treatment of choice for bleeding varices^[8]. Placing a rubber band around the variceal vein induces venous obstruction followed by mucosal inflammation, necrosis, and obliteration of the variceal vein. The single-shot mechanism of the ligation device is inherently inefficient, and makes the procedure tedious. It also requires overtube placement, associated with discomfort and complications^[13-15]. Multiple-band ligation devices make band ligation easier and more efficient, allowing the consecutive application of 5 to 10 bands without removing the endoscope.

Reuse of equipment will always be cheaper than using new equipment. The issue becomes important when patients have to pay for all medical costs themselves and are not covered by a health care plan. The main issue is safety of reusable equipment. There were no band ligator failures or other complications noted in our patients with reloaded equipment. Very occasionally an extra band slipped off while deploying. There were no infection issues in these patients. Reuse of 'disposable' medical equipments may be a source of infection for HBV, HCV, and HIV in less developed countries. We disinfected the disposable items of the ligator with glutaraldehyde according to the standard recommendations and closed in a sealed bag with a label of patient's identification details and stored in an allocated dry place in the endoscopy suite. On arrival of the patient, the bag was opened and the ligator was reloaded with aseptic precautions to be used on the same patient. It is not too difficult to reload the band ligator. The process takes about five minutes.

Variceal eradication was achieved in 70% of the patients enrolled in our eradication program. A wide range of success rates in eradication of esophageal varices has been reported in several studies. In the study by Stiegmann *et al*^[6], variceal obliteration occurred in 27 patients of 64 (42%) while in the study by Lo *et al*^[7] varices were eradicated in 74%. Cost savings of the whole procedure using reloaded band ligator were 58%. Cost saving of the ligators, if reloaded equipment was used, was 95%. The band ligator was virtually free as only the costs of the rubber bands was charged. Rest of the expenses was related to the endoscopy and recovery.

In conclusion, EVL using reloaded polyband ligators for the follow-up sessions on patients undergoing variceal eradication is a cost effective procedure and may be recommended for developing countries.

COMMENTS

Background

Band ligation of esophageal varices is indicated as a primary prophylaxis for large varices and as a secondary prophylaxis for patients who have bled from varices. It is the endoscopic procedure of choice to prevent recurrent variceal hemorrhage and to eradicate varices. It usually requires 3-4 sessions using multiband ligator and applying up to six bands each time. Endoscopic variceal ligation (EVL) is an expensive procedure, especially for patients from lower socioeconomic class in developing countries.

Research frontiers

Instead of using new multiband ligator for each session, reloading the ligator and using it for subsequent sessions on the same patient would substantially reduce the costs.

Related publications

Not much published work related to this aspect is available. We described the method of reloading of the variceal multiple band ligator using hemorrhoidal banding apparatus (letter). *J Pak Med Asso (JPMA)* 2000; 50: 285-286.

Innovations and breakthroughs

Cost saving of the whole procedure using reloaded band ligator was 58% of the cost had new ligator been used. The band ligator is virtually free as only the cost of the rubber bands is charged. Rest of the expenses is related to the endoscopy and recovery.

Applications

EVL using reloaded polyband ligators for the follow-up sessions on patients undergoing variceal eradication is a cost effective procedure and may be recommended for developing countries.

Terminology

Multiband ligator is a device used to ligate esophageal varices, allowing the consecutive application of six rubber bands without removing the endoscope.

Peer review

This article reports Pakistanis experience about EVL using reloaded multiband ligator. I have a great experience about this procedure in my department. It is indicated for the third world countries. This is a nice, simple, and short paper with a clear point.

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RAPID COMMUNICATION

Effect of thermal cutaneous stimulation on the gastric motor activity: Study of the mechanism of action

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Shafik A, Shafik AA, Sibai OE, Shafik IA. Effect of thermal cutaneous stimulation on the gastric motor activity: Study of the mechanism of action. *World J Gastroenterol* 2008; 14(14): 2226-2229 Available from: URL: <http://www.wjgnet.com/1007-9327/14/2226.asp> DOI: <http://dx.doi.org/10.3748/wjg.14.2226>

Abstract

AIM: To investigate the mechanism of action of thermal cutaneous stimulation on the gastric motor inhibition.

METHODS: The gastric tone of 33 healthy volunteers (20 men, mean age 36.7 ± 8.4 years) was assessed by a barostat system consisting of a balloon-ended tube connected to a strain gauge and air-injection system. The tube was introduced into the stomach and the balloon was inflated with 300 mL of air. The skin temperature was elevated in increments of 3°C up to 49°C and the gastric tone was simultaneously assessed by recording the balloon volume variations expressed as the percentage change from the baseline volume. The test was repeated after separate anesthetization of the skin and stomach with lidocaine and after using normal saline instead of lidocaine.

RESULTS: Thermal cutaneous stimulation resulted in a significant decrease of gastric tone $61.2\% \pm 10.3\%$ of the mean baseline volume. Mean latency was 25.6 ± 1.2 ms. After 20 min of individual anesthetization of the skin and stomach, thermal cutaneous stimulation produced no significant change in gastric tone.

CONCLUSION: Decrease in the gastric tone in response to thermal cutaneous stimulation suggests a reflex relationship which was absent on individual anesthetization of the 2 possible arms of the reflex arc: the skin and the stomach. We call this relationship the "cutaneo-gastric inhibitory reflex". This reflex may have the potential to serve as an investigative tool in the diagnosis of gastric motor disorders, provided further studies are performed in this respect.

INTRODUCTION

External stimuli have been shown to affect gastric motility. Centrally acting stressful stimuli produce gastrointestinal motility changes in rats^[1-6], dogs^[7,8], and humans^[9-11]. These actions seem to be mediated through humoral pathways^[12-14]. Thus α - and β -adrenergic blockers are claimed to abolish the inhibition of gastric motility induced by cold pain in humans^[15]. Other investigators suggest that other humoral factors, such as acoustic stress, may be involved in the mediation of the gastrointestinal motor disturbances^[16-19]. Gastric ulcer and acute pancreatitis may also be related to stress^[11,20].

It has been shown that various types of stressors cause a release of the corticotrophin-releasing factor and that intra-cerebroventricular administration of this factor mimics the motor, metabolic, and hemodynamic responses to such stimuli in animals^[21-24]. However, in anesthetized rodents, an increased gastric motility was observed during restraint stress^[25,26], whereas pinching of the skin was accompanied by gastric motor inhibition^[20].

Although skin pinching or stressful cutaneous stimuli have been demonstrated to be associated with gastric motor inhibition^[27,28], the mechanisms involved in this action have not been elucidated in the literature. Therefore, hypothesizing that skin stimulation induces its effect on the gastric motor activity through a reflex action, we conducted the current study.

MATERIALS AND METHODS

Subjects

Thirty-three subjects [20 men and 13 women; mean age 36.7 ± 8.4 (range 26-45) years] were enrolled in this

study after they had given an informed consent. The results of physical examination including neurological assessment were normal. Laboratory work up including blood count, renal and hepatic function tests, as well as electrocardiography were normal. The study was approved by the Faculty of Medicine Review Board and Ethics Committee of Cairo University.

Methods

Thermal cutaneous stimulation (TCS) was performed by means of a thermal pad applied to the skin, and the gastric motor activity was recorded with a barostat. A 6F polyvinyl gastric tube, with multiple side holes 4 to 6 cm from its distal end, was used. A thin compliant polyethylene balloon (London Rubber Industries Ltd, London, UK) was fastened to the distal part of the tube that contained the side holes. The tube had a metallic clip applied to its distal end for fluoroscopic control. It was connected to a strain gauge and a computer-controlled air-injection system (G&J Electronics Inc, Toronto, Ontario). This barostat system keeps the pressure within the balloon constant. Thus, when the gastric tone increases, the air in the bag is withdrawn, and when the tone diminishes, the air rushes into the balloon; hence the pressure in the balloon is kept constant at all times. Using this technique, the gastric tone could be assessed by recording the balloon volume variations, expressed as the percentage change from the baseline volume.

The tube was introduced into the stomach through the nose. The tests were performed 20 min later so that the stomach would have adapted to the inserted catheter. The balloon was then inflated with 300 mL of air. Thermal stimulation of the skin was induced by a thermal pad applied to the skin of the upper arm and connected to a thermostat.

The skin temperature was recorded at rest. The pad temperature was then elevated in increments of 3°C above the resting skin temperature up to 49°C or the highest tolerable temperature. Throughout the period of successive skin temperature elevation, the gastric wall tone was simultaneously assessed by measuring the variations in the balloon volume, expressed as the percentage change from the baseline volume. We calculated the latency which is the period between the start of thermal skin stimulation and the beginning of the gastric tone response.

To define whether the effect of thermal cutaneous stimulation (TCS) on the stomach was a direct or a reflex action, the following test was performed.

Cutaneous and gastric anesthetization

The aforementioned test was repeated after individual anesthetization of the skin and stomach. The skin area, over which the thermal pad was applied, was anesthetized by injection of 3 mL of 2% lidocaine mixed with 3 mL of normal saline; the injection was performed at multiple points in the skin under the pad. The gastric tone response to TCS, as aforementioned, was recorded after 20 min and 3 h later when the anesthetic effect had waned. The stomach was then anesthetized by endoscopic injection of 30 mL of 2% lidocaine in 70 mL of normal saline. The

Table 1 Change in the gastric tone in response to the different degrees of thermal cutaneous stimulation (mean \pm SD)

Skin temperature (°C)	Basal tone (% of baseline volume)	
	Mean	Range
37 (basal)	0	0
40	48.2 \pm 6.4	40-56
43	57.3 \pm 5.1	52-63
46	69.7 \pm 3.3	66-74
49	78.6 \pm 4.1	76-83

injection was performed at multiple points in the stomach wall. The gastric tone response to TCS was then registered after 20 min and 3 h later. The aforementioned tests were repeated using normal saline instead of lidocaine.

RESULTS

The study was completed without any adverse side effects during or after the tests. During TCS, all of the subjects showed a significant decrease in the gastric tone which varied from 40% to 83% (mean 61.2% \pm 10.3%) of the baseline volume according to the degree of TCS (Figure 1, Table 1). There was a progressive decrease in the gastric tone with increasing TCS (Figure 1, Table 1). Gastric tone decline was greater in men than in women but the difference was not significant ($P > 0.05$). Also, there was no significant difference in the gastric tone decrease between the younger and older subjects. The latency varied from 20.6-28.8 ms (mean 25.6 \pm 1.29). It decreased with increasing TCS. There was no significant difference in the latency when we compared men to women or younger to older subjects.

Effect of TCS on the gastric tone after individual cutaneous and gastric anesthetization

TCS performed 20 min after individual anesthetization of the skin or stomach produced no significant changes in the gastric tone (Figure 2). Three hours later, when the anesthetic effect had waned, the TCS caused a decrease in gastric tone similar to that before anesthetization ($P > 0.05$). When the above tests were repeated using saline instead of lidocaine, the gastric tone response was similar to that before saline application ($P > 0.05$).

Upon repetition of the aforementioned tests in the same subject, similar results were obtained with no significant differences.

DISCUSSION

It is established that centrally acting stressful stimuli induce changes in the gastrointestinal motility^[1-11]. These changes are suggested to occur through humoral pathways as supported by the release of β -endorphin and catecholamines into the peripheral circulation during stress^[12-15]. In agreement with such hypothesis, naloxone or a combination of α - and β -adrenergic blockers abolish gastric motility inhibition induced by cold pain. Yet, this hypothesis can be ruled out because the aforementioned drugs have no effect on the migrating duodenal activity

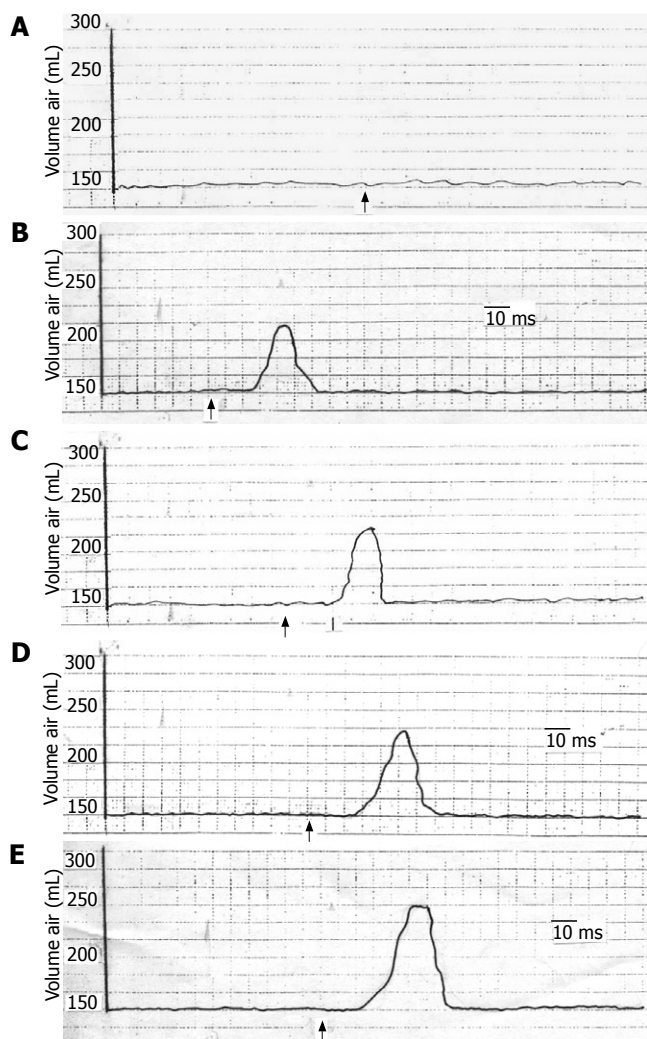


Figure 1 Decrease in the gastric tone in response to thermal cutaneous stimulation (arrow). (A) 37°C (basal); (B) 40°C; (C) 43°C; (D) 46°C; and (E) 49°C.

induced by labyrinthine stimulation in humans^[24-28] or on acoustic stress-induced inhibition of gastric motility in dogs^[27]. This suggests that other factors may be involved in the mediation of gastrointestinal motor disturbances induced by centrally acting stimuli.

The current study demonstrated that TCS affected inhibition of gastric motor activity which progressively increased on incremental enhancement of TCS. This effect was abolished on anesthetization of either the stimulated cutaneous area or the stomach. The inhibited gastric motor activity presumably denotes gastric wall relaxation and gastric dilatation. It seems that the stomach dilates on stress to avoid gastric stimulation that might result in vomiting.

The current findings led to the assumption that the inhibited gastric motor activity in response to cutaneous stressful condition is mediated through a reflex pathway. This hypothesis is evidenced by the findings that, with individual anesthetization of the suggested 2 arms of the reflex arc, i.e. the skin and the stomach, the gastric response was absent. Saline on the other hand did not give rise to such effect. The response returned after the anesthetic condition had worn off. Furthermore, the reproducibility of the effect points to the constancy

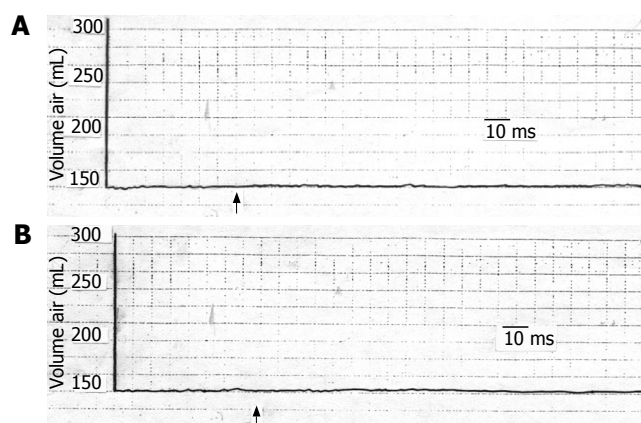


Figure 2 Response of the gastric tone to thermal cutaneous stimulation (arrow) at 46°C 20 min after separate anesthetization of the skin (A) and the stomach (B).

of the results. We call the suggested reflex response of the stomach to cutaneous stimulation, the “cutaneo-gastric inhibitory reflex (CGIR)”. It may be argued that this effect could be humoral as already mentioned by investigators^[10-18]. However, if the effects of cutaneous stimulation on the stomach were humoral, it would not vanish with either gastric or cutaneous anesthetization as has been shown in the current findings. Meanwhile, the effect of centrally acting stressful stimuli on the stomach^[1-6] cannot be ignored, albeit that this role alone does not seem to explain the non-response of the stomach to stimulation of the anesthetized skin.

It seems that TCS activates the cutaneous nerve endings which send impulses along the afferent fibers to the spinal cord. Impulses from the spinal cord are in turn transmitted along efferent fibers to the stomach, inhibiting its motor activity.

The point that needs to be discussed is: what could be the possible clinical significance of the CGIR? It is suggested that the CGIR might be of diagnostic significance in gastric motility disorders. Diminished gastric tone response to TCS would indicate a defect in the reflex pathway, such as gastric musculature or nerve damage resulting from a disease of the peripheral nerves, spinal nerve roots or spinal cord or from a central lesion. Significant prolongation of the latency of the CGIR on the other hand may indicate a disorder of the reflex arc. We believe that the CGIR may be incorporated as an investigative tool in the study of patients with gastric disorders after it has been further studied in various pathologic gastric lesions. The reflex assesses the integrity of the gastric motor activity.

In conclusion, TCS results in decrease of the gastric motor activity which apparently leads to gastric wall relaxation. The decrease in gastric tone upon TCS postulates a reflex relationship which was absent on individual anesthetization of the assumed two arms of the reflex arc: the skin and the stomach. We call this relationship the CGIR. This reflex may prove to be of diagnostic significance in gastric motor disorders and have the potential to serve as an investigative tool, provided further studies are performed to validate the current results.

ACKNOWLEDGMENTS

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COMMENTS

Background

External stimuli have been shown to affect gastric motility. Centrally acting stressful stimuli produce gastrointestinal motility changes in rats, dogs, and humans. These actions seem to be mediated through humoral pathways. Thus α - and β -adrenergic blockers are claimed to abolish the inhibition of gastric motility induced by cold pain in humans. Other investigators suggest that other humoral factors such as acoustic stress may be involved in the mediation of the gastrointestinal motor disturbances. We hypothesized that skin stimulation induces its effect on the gastric motor activity through a reflex action. This hypothesis was investigated in the current study.

Research frontiers

It is established that centrally acting stressful stimuli induce changes in the gastrointestinal motility. Other factors may be involved in the mediation of gastrointestinal motor disturbances induced by centrally acting stimuli.

Innovations and breakthroughs

The point that needs to be discussed is: what could be the possible clinical significance of the cutaneo-gastric inhibitory reflex (CGIR)? It is suggested that the CGIR might be of diagnostic significance in gastric motile disorders. Diminished gastric tone response to thermal cutaneous stimulation (TCS) would indicate a defect in the reflex pathway, such as gastric musculature or nerve damage resulting from a disease of the peripheral nerves, spinal nerve roots or spinal cord or from a central lesion. We believe that the CGIR may be incorporated as an investigative tool in the study of patients with gastric disorders after it has been further studied in various pathologic gastric lesions. The reflex assesses the integrity of the gastric motor activity.

Peer review

The authors had asked a simple question, namely whether gastric tone responds to cutaneous stimulation with heat. The answer is straight forward: Heat application leads to gastric relaxation and this effect can be abolished by intracutaneous and intragastric injections of lidocaine.

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RAPID COMMUNICATION

DNMT3B 579 G>T promoter polymorphism and risk of esophagus carcinoma in Chinese

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CONCLUSION: DNMT3B 579 G>T polymorphism may not be a stratification marker to predict the susceptibility to EC, at least in Chinese. DNMT3B promoter SNP is diverse in ethnic populations.

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Key words: Esophagus cancer; DNMT3B; Methylation; Polymorphism

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Abstract

AIM: To investigate the relationship between 579 G>T polymorphisms in the DNMT3B gene, which is involved in de novo methylation and associated with the risk of esophagus cancer (EC) in Chinese.

METHODS: DNMT3B 579 G>T genotypes were determined by PCR-RFLP in 194 EC patients and 210 healthy controls matched for age and sex, who did not receive radiotherapy or chemotherapy for newly diagnosed and histopathologically confirmed EC.

RESULTS: In control subjects, the frequency of T/T and G/T genotypes, and T and G alleles was 80.5%, 19.0%, 90.0% and 10.0%, respectively. The distribution of genotypes and allelotypes in the EC patients was not significantly different from that in the controls. When stratified by sex and age, there was still no significant association between the risks of EC and GT and GG genotypes. This study also showed a distinct difference in the distribution of DNMT3B and single nucleotide polymorphism (SNP) between Chinese and Koreans.

INTRODUCTION

Esophagus carcinoma (EC) is one of the most common malignancies and the main cause of cancer-related death in the world. Because symptoms typically remain absent until late in the course of disease, most cancers are detected at an advanced stage when prognosis is poor. Therefore, it is important to investigate the genetic and epigenetic variation in susceptibility to esophagus carcinogenesis and identify the markers that will facilitate identification of individuals at risk of esophagus carcinogenesis.

DNA methylation is a major epigenetic modification involving the addition of a methyl group to the 5' position of a cytosine in a CpG dinucleotide. A number of studies suggested that aberrant DNA cytosine methylation may play an important role in carcinogenesis^[1-5]. DNMT3A and DNMT3B are required for the establishment and maintenance of genomic methylation patterns and proper murine development^[6-9]. Both genes are up-regulated to different degrees in some malignancies, including colon cancer and EC^[10-14]. Recently, several candidate single nucleotide polymorphisms (SNPs) in the DNMT3B gene have been deposited in public databases. Although the functional effects of these polymorphisms have not been elucidated, some studies showed that some of these variants may influence the DNMT3B activity on DNA methylation,

thereby modulating the susceptibility to lung cancer, breast cancer and gastric cardiac adenocarcinoma^[15-17]. The DNMT3B gene contains a single G>T SNP in the transcription start site of the promoter region (-579 bp from exon 1B), and this probably affects gene function^[18]. Some studies suggested that DNMT3B -579 G>T may modify susceptibility to tumors. Although conflicting results have been reported in different tumor types, the heterozygous genotypes have a significantly reduced risk of developing lung and colon cancer^[19-21]. However, no report on the association between this allele and the development of EC is available. This study was to investigate the association between this polymorphism and EC in Chinese.

MATERIALS AND METHODS

Study population

This case-control study included 194 EC patients and 210 healthy controls. EC was histopathologically confirmed in the 194 patients during surgery at the Zhongda Hospital of Southeast University and Tumor Hospital, Nanjing, China. The control subjects were selected from cancer-free subjects who visited the same hospital for a regular physical examination and volunteered to participate in the epidemiology survey during the same period. We defined a healthy subject as a person free of disease (including no history of cancer) at health check-up. The controls were matched for age and sex with the patients (Table 1). All patients and controls were ethnically Chinese and resided in Jiangsu Province or in its surrounding regions.

DNA extraction

Five milliliters of venous blood was drawn from each subject into vacuum tubes containing EDTA and stored at 4°C. Genomic DNA was extracted within one week after sampling by proteinase K digestion and salted out as previously described^[19].

DNMT 3B genotyping

Transition from G to T of the DNMT3B SNP creates a *PvuII* restriction site, which can be exploited for genotyping by PCR and subsequent restriction fragment length polymorphism (RFLP) analysis. PCR was performed in a volume of 25 µL containing 100 ng of DNA template, 10 × PCR master mix (Promega, USA), and 10 pmol/L each of sense primer (5'-GAGGTCTCATATGCCTAGG-3') and antisense primer (5'-GGGAGCTCACCTTCTAGAAA-3'). For PCR amplification, an initial denaturation at 94°C for 5 min was followed by 30 cycles at 94°C for 30 s, at 57°C for 30 s, at 72°C for 30 s, and a final extension at 72°C for 7 min. The PCR products were digested overnight with 5 units of *PvuII* (New England Biolabs, Beverly, Mass.) at 37°C and separated on 2% agarose gels. RFLP bands were visualized under UV light with ethidium bromide staining. The DNMT3B T/T genotype was expected to show two DNA bands at the positions of 132 bp and 93 bp, whereas the G/G genotype was expected to show a single band (225 bp), and the heterozygote was expected to have three bands (225 bp, 132 bp, 93 bp). For quality control, genotyping analysis was performed blindly with respect to case/control status and repeated twice for all subjects.

Table 1 Distribution of selected variables in esophagus cancer patients and control subjects *n* (%)

Variables	Patients (<i>n</i> = 194)	Control (<i>n</i> = 210)	<i>P</i>
Age (yr)			> 0.05
< 40	1 (0.5)	2 (0.9)	
40-60	117 (60.3)	110 (52.4)	
61-80	76 (39.2)	98 (46.7)	
Sex			> 0.05
Male	150 (77.3)	146 (69.5)	
Female	44 (22.7)	64 (30.5)	

DNA sequencing analysis

To confirm the genotyping results, selected PCR-amplified DNA samples were examined by DNA sequencing. The PCR fragments were recovered from agarose gel followed by purification with a DNA clean-up kit (Wizard SV Gel and PCR Clean-up System, Promega). DNA sequences of the PCR products were determined using the PCR sense primer with an Applied Biosystems model 377 sequencer (PE Applied Biosystems, Warrington, UK). The results of genotyping were 100% concordant.

Statistical analysis

Patients and controls were compared using Student's *t*-test for continuous variables and chi square (χ^2) test for categorical variables. Hardy-Weinberg equilibrium was tested with a goodness-of-fit χ^2 test with one degree of freedom to compare the observed genotype frequencies with the expected genotype frequencies among the subjects. Comparison of the DNMT3B genotype and allelotype distribution in the study groups was performed by means of two-sided contingency tables using χ^2 test or Fischer's exact test. The odds ratio (OR) and 95% confidence interval (CI) were calculated using an unconditional logistic regression model and adjusted for age and gender accordingly. *P* < 0.05 was considered statistically significant.

RESULTS

The demographics of the cases and controls enrolled in this study are shown in Table 1. There were no significant differences in the mean age and sex distribution between cases and controls, suggesting that the matching based on these two variables was adequate. There was no evidence of a deviation from Hardy-Weinberg equilibrium among the cases or controls. The mean age of the patients and controls was 59.6 years (\pm 10.2 years; range, 34-80 years) and 59.6 years (\pm 10.2 years; range, 34-80 years), respectively.

All the patients and controls were successfully genotyped for the DNMT3B polymorphism (Figure 1). The genotyping by PCR-RFLP analysis was completely confirmed by DNA sequencing analysis, and the results of PCR-RFLP genotyping and sequencing analysis were also 100% concordant (Figure 2). The distribution of DNMT3B 579 G>T polymorphism was in Hardy-Weinberg equilibrium. The frequency of G allele in control subjects (0.10) was different from that in the previous

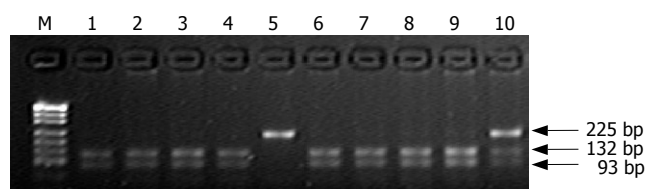


Figure 1 PCR-based restriction fragment length polymorphism genotyping of DNMT3B 579 G>T. Lanes 1-4, 6-9: TT variants; lane 5: GG wild type; lane 10: GT heterozygote.

Table 2 DNMT3B genotype and allele frequency in Chinese and Koreans

	TT	GT	GG	G allele frequency (%)
Chinese	169 (80.5)	40 (19.0)	1 (0.5)	10.0
Korean	153 (61.7)	91 (36.7)	4 (1.6)	20 ^b

^b*P* < 0.01 vs Chinese.

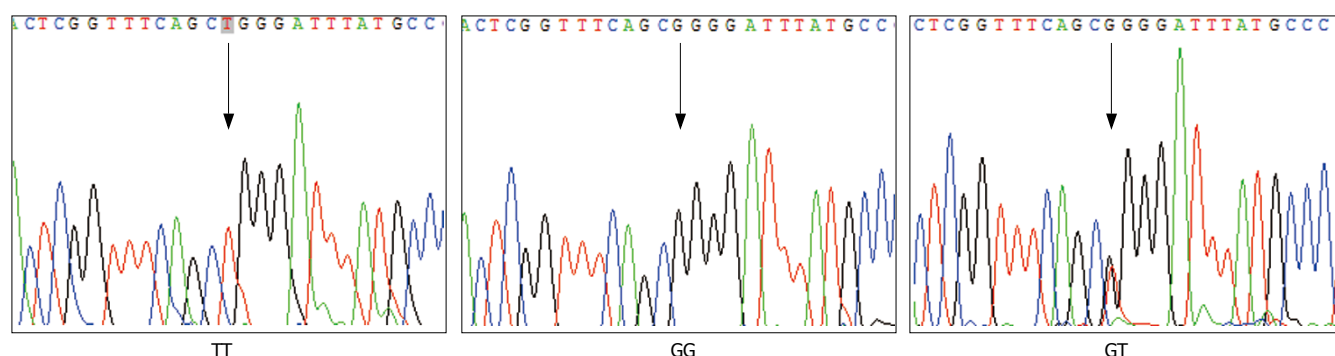


Figure 2 Sequencing results for each of the PCR products from different genotypes. The SNP sites are indicated by the arrowhead. The results were completely matched to the corresponding results derived from PCR-RFLP genotyping.

study among Koreans (0.20)^[21]. The distributions of DNMT3B 579 G>T genotypes in Chinese and Koreans are shown in Table 2.

The distributions of DNMT3B 579 G>T genotypes in controls and patients are shown in Table 3. The genotype distributions of both polymorphisms in the controls were in Hardy-Weinberg equilibrium. No significant deviation was observed in the genotype distributions of both polymorphisms between overall esophagus cancer patients and controls. Then we stratified the results by sex and age, patients and controls were found to be slightly different with respect to the genotype distribution (Table 4). Combined GG and GT genotypes were found to have a little higher OR in male esophagus cancer patients and the group under the age of 59 years (males: OR 1.35 ; 95% CI, 0.76-2.39; under 59: OR 1.47 ; 95% CI, 0.74-2.90). However, combined GG and GT genotypes showed no significant association between DNMT3B 579 G>T polymorphism and the risk of esophagus cancer.

DISCUSSION

Single nucleotide polymorphism (SNP) is the most common form of human genetic variation, and may contribute to an individual's susceptibility to cancer. Studies suggested that some variants in the promoter region of genes may affect either the expression or activity levels of enzymes^[1,18,22] and therefore may be mechanistically associated with cancer risk. It has been recently shown that SNP of the DNMT3B promoter 579 G>T (from exon 1B transcription start site) decreases the susceptibility of an individual to lung and colon cancer^[20,21], suggesting that DNMT3B promoter 579 G>T

Table 3 DNMT3B genotype and allele frequency in patients and control subjects and their association with esophagus cancer *n* (%)

	Case patients (<i>n</i> = 194)	Control subjects (<i>n</i> = 210)	OR (95% CI)
DNMT3B 579 G>T			
TT	151 (77.8)	169 (80.5)	
GT	43 (22.2)	40 (19.0)	1.17 (0.73-1.90)
GG	0 (0)	1 (0.50)	
G allele (%)	11.1	10.0	

Table 4 Stratification analysis of DNMT3B 579 G>T genotype frequencies in esophagus cancer patients and controls, adjusted OR (95% CI)

Variable	TT genotype Case/control	GT + GG genotype Case/control	Odds ratios of GT + GG genotype
Age			
< 60	81/89	24/18	1.47 (0.74-2.90)
≥ 60	70/80	19/23	0.94 (0.48-1.88)
Sex			
Male	116/120	34/26	1.35 (0.76-2.39)
Female	35/49	9/15	0.84 (0.33-2.14)

polymorphism can be used as a risk factor for cancer to evaluate the population susceptible to tumors. However, it was also reported that there is no association between polymorphism of 579 G>T and head and neck squamous cell carcinoma^[23]. However, to the best of our knowledge, the relative significance of SNP in the genetic susceptibility to esophagus cancer has not yet been disclosed. In the

current study, we investigated the influence of DNMT3B polymorphisms on the risk of esophagus cancer in a hospital-based case-control study.

This is the first study of DNMT3B polymorphism in esophagus cancer. We investigated the influence of 579 G>T polymorphism in the DNMT3B gene on the risk of esophagus cancer. Individuals carrying G allele in the DNMT3B gene were found to have a nearly consistent risk of EC compared with those carrying T allele. Then we stratified the results by sex and age, patients and controls. Combined GG and GT genotypes showed no significant association between DNMT3B 579 G>T polymorphism and risk of esophagus cancer, suggesting that 579 G>T polymorphism in the DNMT3B gene cannot be used as a marker of genetic susceptibility to esophagus cancer even in young individuals. Our study showed that DNMT3B polymorphism was not associated with the risk of esophagus carcinoma, at least in the study population, although other studies reported a decreased risk of lung and colon cancer in those harboring G allele. Since the different variants of DNMT3B may alter catalytic activity and are expressed in a tissue specific manner^[24-27] and the repression of DNMT3B activity does not result in re-expression of all hypermethylated tumor suppressor genes in some cell systems^[28-31], it is therefore important to explore the complex interplay of DNMTs in different tumor types.

In this study, a distinct difference was found in the distribution of DNMT3B SNP between Chinese and Koreans. However, few G/G genotypes were found in both populations. Additionally, the frequency of G/T genotype in Chinese was lower than that in Koreans. The great diversity in DNMT3B SNP distribution in different ethnic populations remains unknown.

In conclusion, the DNMT3B gene may not be involved in the development of esophagus cancer. Further studies with a larger sample are required to confirm our findings, to understand the role of DNMT3B polymorphisms in determining the risk of esophagus cancer, and to clarify the association of DNMT3B polymorphism with esophagus cancer in different ethnic populations.

COMMENTS

Background

DNA methylation is a major epigenetic modification involving the addition of a methyl group to the 5' position of a cytosine in CpG dinucleotide. DNMT3A and DNMT3B are required for the establishment and maintenance of genomic methylation patterns. Single nucleotide polymorphisms (SNPs) in the DNMT3B gene may influence DNMT3B activity on DNA methylation, thereby modulating the susceptibility to some cancer.

Research frontiers

Some variants in the promoter region of genes may affect either the expression or activity levels of enzymes and therefore may be mechanistically associated with cancer risk. It has been recently reported that SNP of the DNMT3B promoter 579 G>T (from exon 1B transcription start site) decreases the susceptibility of an individual to lung and colon cancer. However, it was also reported that there is no association between polymorphism of 579 G>T and head and neck squamous cell carcinoma. The relative significance of SNP in genetic susceptibility of an individual to cancer is diverse in different populations.

Innovations and breakthroughs

It is important to investigate the genetic and epigenetic variation in susceptibility to

esophagus carcinogenesis and identify markers that will facilitate identification of individuals at risk of esophagus carcinogenesis. Although no significant association was found between DNMT3B 579 G>T polymorphism and risk of esophagus cancer, this is the first study of DNMT3B polymorphism in esophagus cancer. This study also showed the significance of great diversity in DNMT3B SNP distribution in different ethnic populations and their susceptibility to cancer.

Applications

A distinct difference was found in the distribution of DNMT3B SNP between Chinese and Koreans in this study. The significance of great diversity in DNMT3B SNP distribution in different ethnic populations remains unknown. These results suggest that the DNMT3B gene may not be involved in the development of esophagus cancer. Future studies of other DNMT3B sequence variants and their biologic function are needed to understand the role of DNMT3B polymorphisms in determining the risk of esophagus cancer.

Peer review

The association between DNMT3B 579 G>T polymorphism and the risk of esophagus cancer in Chinese was studied. The results show that the DNMT3B 579 G>T polymorphism was not associated with the risk of esophagus carcinoma, at least in the study population. This study also showed the significance of great diversity in DNMT3B SNP distribution in different ethnic populations and their susceptibility to cancer.

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Anti-sense oligonucleotide labeled with technetium-99m using hydrazinonictinamide derivative and N-hydroxysuccinimidyl S-acetylmercaptoacetyltriglycine: A comparison of radiochemical behaviors and biological properties

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radiochemical behaviors and biological properties than ^{99m}Tc -HYNIC-ASON. ^{99m}Tc -MAG₃-ASON is a potential radiopharmaceutical agent for *in vivo* application.

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Key words: Anti-sense oligonucleotide; Radiolabeling; Technetium-99m; N-hydroxysuccinimidyl S-acetylmercaptoacetyltriglycine; Hydrazinonictinamide derivative

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Abstract

AIM: To explore and compare the radiochemical behavior and biological property of anti-sense oligonucleotide (ASON) labeled with technetium-99m using N-hydroxysuccinimidyl S-acetylmercaptoacetyltriglycine (NHS-MAG₃) and hydrazinonictinamide derivative (HYNIC).

METHODS: After HYNIC and NHS-MAG₃ were synthesized, ASON was labeled with technetium-99m using HYNIC and NHS-MAG₃ as a bifunctional chelator. The *in vivo* and *in vitro* stability, binding rates of labeled compounds to serum albumen, biodistribution of ^{99m}Tc -MAG₃-ASON and ^{99m}Tc -HYNIC-ASON in BALB/C mouse and its HT29 tumor cellular uptake were compared.

RESULTS: The labeling efficiency and stability of ^{99m}Tc -MAG₃-ASON were significantly higher than those of ^{99m}Tc -HYNIC-ASON ($P = 0.02$, and $P = 0.03$, respectively). ^{99m}Tc -MAG₃-ASON had a significantly lower rate of binding to serum albumen than ^{99m}Tc -HYNIC-ASON ($P < 0.05$). In contrast to ^{99m}Tc -HYNIC-ASON, the biodistribution of ^{99m}Tc -MAG₃-ASON was significantly lower in blood, heart, liver and stomach ($P < 0.05$), slightly lower in intestines and spleen ($P > 0.05$) and significantly higher in lung and kidney ($P < 0.05$). The HT29 tumor cellular uptake rate of ^{99m}Tc -MAG₃-ASON was significantly higher than that of ^{99m}Tc -HYNIC-ASON ($P < 0.05$).

CONCLUSION: ^{99m}Tc -MAG₃-ASON shows superior

Li YC, Tan TZ, Zheng JG, Zhang C. Anti-sense oligonucleotide labeled with technetium-99m using hydrazinonictinamide derivative and N-hydroxysuccinimidyl S-acetylmercaptoacetyltriglycine: A comparison of radiochemical behaviors and biological properties. *World J Gastroenterol* 2008; 14(14): 2235-2240 Available from: URL: <http://www.wjgnet.com/1007-9327/14/2235.asp> DOI: <http://dx.doi.org/10.3748/wjg.14.2235>

INTRODUCTION

Different drugs can be used in anti-sense therapy, among which synthetic anti-sense oligonucleotide (ASON) is used to bind to deoxyribonucleic acid (DNA) translation or transcription in a sequence-specific manner and interfere with the expression of oncogene. However, it is still difficult for ASON to target tumor cells and transport across cell membrane. Besides, because of multi-gene expressions in tumor cells, inhibition of any single target gene is not sufficient to inhibit tumor growth. Radio-labeled ASON targeting specific oncogenes can overcome these problems by direct inhibition of anti-sense and radiation damage. The curative effect of radionuclide anti-sense therapy is closely related to the labeling efficacy of ASON and the characteristics of labeled compounds. In contrast to ^{188}Re , ^{186}Re , ^{90}Y [1-3], hydrazinonictinamide derivative (HYNIC) and N-hydroxysuccinimidyl S-acetylmercaptoacetyltriglycine (NHS-MAG₃), as a bifunctional chelator, have been known to help label

ASON^[4-6] with ^{99m}Tc. However, few reports are available on the comparison of both chelators. This study was to compare the radiochemical behaviors and biological properties of ASON labeled with technetium-99m using NHS-MAG₃ and HYNIC.

MATERIALS AND METHODS

Materials

BALB/c nude mice at the age of 6-8 wk, weighing 17-22 g, were obtained from West China Experimental Animal Center. Human colon carcinoma HT29 cell line was obtained from the Laboratory of West China Hospital. HT29 cells were incubated in RPMI 1640 supplemented with 10% fetal calf serum, 100 U/mL penicillin, 100 µg/mL streptomycin. Fifteen-mer phosphorothioate ASON (5'-NH₂-FACGTTGAGGGGCAT-3', F is adenosine sulfurised), which is complementary to the translation start site of c-myc mRNA, was purchased from GibcoBRL (USA). ^{99m}TcO₄⁻ (37 TBq/L) was obtained from Chengdu Gaotong Isotope Corporation (Chengdu, China). Sephadex G25 was from Pharmacia Fine Chemicals A.B (Uppsala, Sweden). C₁₈ Sep-Pak reversed-phase column was a product from Waters Company (Milford, USA). CRC-15R dose calibrator was from Capintec Company (Ramsey, New Jersey, USA). FH463A automatic scaler was supplied by Beijing Nuclear Instrument Company (Beijing, China). Unity Inova-400 nuclear magnetic resonator was from Varian Company (USA). UV-2100 spectrophotometer was from Beckman Company (Cotati, California, USA). Frozen desiccator was from Marathon Electric Company (New York, USA). CO₂ incubator was from Sanyo Company (Japan). Centrifuge was from Beckman Company (Cotati, California, USA).

Synthesis of HYNIC and NHS-MAG₃

HYNIC was synthesized as previously described^[7] (Figure 1). The end product was purified by recrystallization in isopropyl alcohol and the yield was 75%. The synthesis process of NHS-MAG₃ has been described elsewhere^[6] (Figure 2). The melting temperature of the end product was 140°C-155°C, the yield was 80%. The content was 2.38 ppm (S, 3H, SCOCH₃), 2.80 ppm (S, 4H, succinimidyl), 3.68-3.80 ppm (M, 8H, COCH₂) and 8.20-8.38 ppm (M, 3H, NHCO), respectively, by nuclear magnetic resonance spectroscopy.

^{99m}Tc labeling ASON via HYNIC

ASON (2 mg/mL) buffer was dissolved in 2 mol/L NaCl, 0.5 mol/L NaHCO₃ and 2 mmol/L ethylenediamine tetraacetic acid (EDTA), and HYNIC (10 mg/mL) was dissolved in dimethylformamide (DMF). In 45°C water bath, 31 µL HYNIC and 2 mmol/L EDTA were gradually dropped into a 25 µL ASON solution at the molar ratio of 25:1. The reaction system was filtered through a Sep-Pak C18 reversed-phase column (10 mm × 5 mm) in 60% methanol to remove HYNIC not binding to ASON. HYNIC-ASON was collected with a UV-2100 spectrophotometer and frosted to dry powder for storage. HYNIC-ASON dry powder was labeled on d 15, 30 or 60, respectively. Ten µg HYNIC-

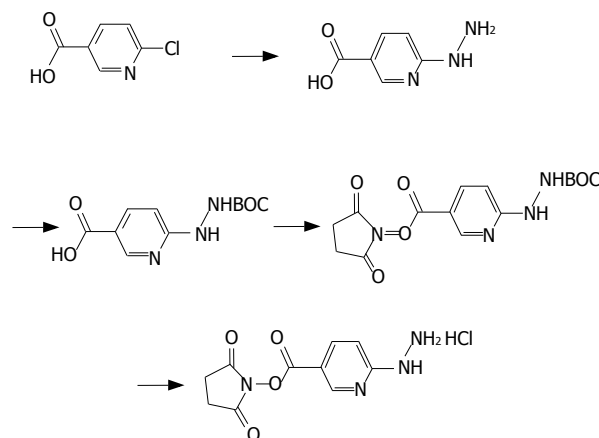


Figure 1 Synthesis of HYNIC.

ASON powder was dissolved in a 0.5 mL tricine solution (70 mg/mL). SnCl₂·2H₂O solution (1 mg/mL) was dissolved in 0.1 mol/L HCl at room temperature. HYNIC-ASON solution, 25 µL SnCl₂·2H₂O solution and 0.2 mL ^{99m}TcO₄⁻ containing a radioactivity of 370, 740 or 1480 MBq were mixed uniformly. After stored for 30 min at room temperature, the mixture was eluted and purified through a Sep-Pak C18 reversed-phase column (10 mm × 5 mm) in 60% methanol, and ^{99m}Tc-HYNIC-ASON solution was collected. Chromatographic assay was performed in both solution systems before and after purification to detect the labeling efficacy and radiochemical purity of ^{99m}Tc-HYNIC-ASON, where Xihua I filter paper as a sustentaculum was developed with 85% methanol as a developer.

^{99m}Tc labeling ASON via NHS-MAG₃

Twenty-five microliter ASON (2 mg/mL, dissolved in 0.25 mol/L NaHCO₃ and 1 mol/L EDTA, pH = 8.5) was mixed with 42 µL NHS-MAG₃ (10 mg/mL, dissolved in dimethylsulphoxide) at the molar ratio of 1:25. The mixture reacted at room temperature in the dark for 15 min. Any free NHS-MAG₃ was removed through Sep-Pak C18 reversed-phase column (10 mm × 5 mm) in 60% methanol. The bound MAG₃-ASON was collected with a spectrophotometer and frosted to dry powder for storage. The target-bound complex, dry powder on d 15, 30 or 60 at room temperature, was labeled. Fifty µL MAG₃-ASON (1 mg/mL) in re-distilled water was mixed with 10 µL NaHCO₃ (0.5 mol/L)-sodium tartrate (50 mg/mL) buffer (pH = 9.2). Ten µL SnCl₂·2H₂O fresh solution (1 mg/mL, dissolved in 0.1 mol/L HCl) was dropped into MAG₃-ASON at room temperature and mixed uniformly. At last, 0.2 mL ^{99m}TcO₄⁻ (containing a radioactivity of 370, 740 or 1480 MBq) was added into the above solutions, respectively. After 15 min, ^{99m}Tc-ASON-MAG₃ was purified on Sephadex G25 column (250 mm × 5 mm) in an ammonium acetate solution (0.25 mol/L, pH = 5.2) and collected. Chromatographic assay was performed in both specimens before and after purification to evaluate the labeling efficiency and radiochemical purity. A system was demanded to develop Xihua I filter paper in 85% methanol.

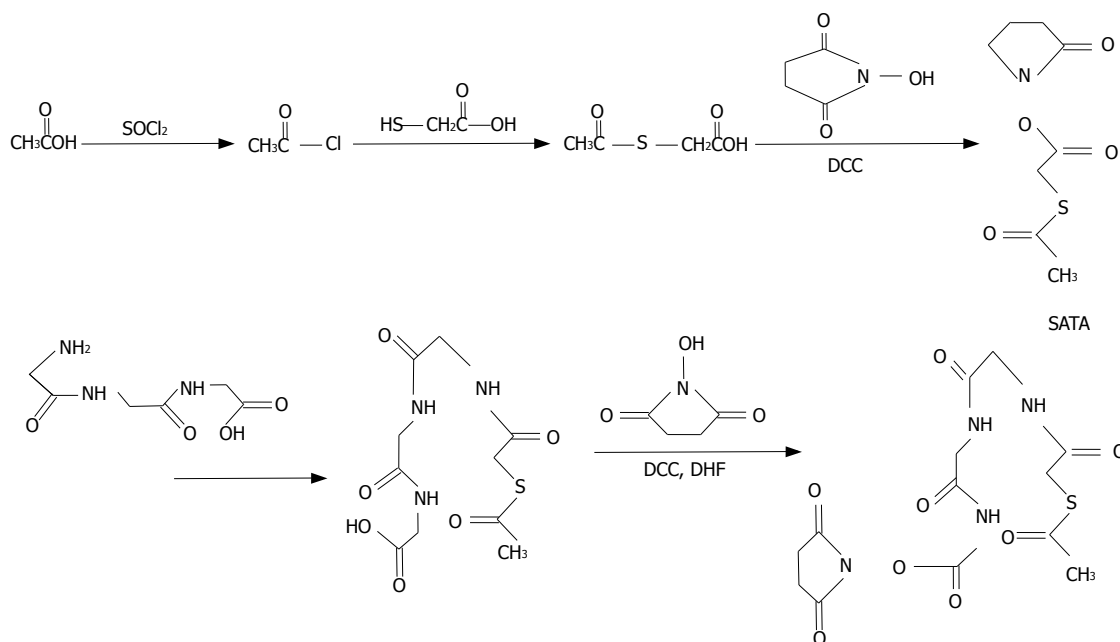


Figure 2 Synthesis of NHS-MAG3. S-acetylthioglycolic acid N-hydroxysuccinimide ester (SATA) was initially synthesized from acetic acid. S-acetyl MAG3 was obtained by the reaction of SATA and triglycine. NHS-MAG3 was produced with the coupling of S-acetyl MAG3 and N-hydroxybutyrylimine.

Stability of labeled compounds

The stability of labeled compounds was assessed for 1, 2 and 4 h, respectively, at room temperature, by measuring the radiochemical purity on Xihua I filter paper that was developed in 85% methanol.

Test for plasma protein binding in rabbits

Three hundred and seventy mL MBq ^{99m}Tc -HYNIC-ASON or ^{99m}Tc -MAG₃-ASON was mixed with 2 mL anti-coagulated rabbit fresh plasma for 6 cuvettes. After incubated at 37 °C for 2 h, the mixture was mixed with 5 mL trichloroacetic acid (250 g/L) and centrifuged for 5 min at $1200 \times g$. The precipitate was washed twice with 2 mL trichloroacetic acid (250 g/L) and the supernatant was collected. The radioactivity of precipitate and supernatant was measured, respectively. The binding rate of ^{99m}Tc -HYNIC-ASON or ^{99m}Tc -MAG₃-ASON to rabbit plasma protein was calculated by the following formula: Binding rate (%) = radioactivity of precipitate/radioactivity of precipitate and supernatant.

Tissue distribution of labeled compounds in BALB/c mice

^{99m}Tc -HYNIC-ASON or ^{99m}Tc -MAG₃-ASON (0.2 mL, 148 KBq) was separately injected into the tail veins of 20 BALB/c nude mice (age: 6-8 wk, body weight: 17-22 g) which were randomly divided into four groups (5 in each group). Mice in each group were sacrificed at 0.5, 1, 2 and 4 h, respectively, after injection of ^{99m}Tc -HYNIC-ASON or ^{99m}Tc -MAG₃-ASON. Blood, heart, lungs, liver, kidneys, spleen, stomach, intestine and muscles were removed and weighed. The tissue uptake rate of labeled compounds was calculated according to the following equation: tissue uptake rate (%ID/g) = radioactivity of per gram of wet tissue weight/radioactivity of wet tissue injected into the body. The results were expressed as percentage of radioactivity within per gram of wet tissue.

Cellular uptake of labeled compounds

Human colon carcinoma HT29 cells were incubated with RPMI 1640 medium containing 10% fetal calf serum, 100 U/mL penicillin and 100 µg/mL streptomycin. Tumor cells were cultured in 80 wells of 96-well plates (1.5×10^6 cells/well). It took about 24 h for cells to adhere to wells. The culture medium was pipetted and 2 mL serum-free RPMI 1640 medium containing 74 KBq ^{99m}Tc -HYNIC-ASON or ^{99m}Tc -MAG₃-ASON was added to each of the 80 wells. The cells were incubated in a humidified incubator containing 50 mL/L CO₂ at 37°C for 10, 20, 40, 60 and 120 min, respectively. Each well was rinsed 3 times with RPMI 1640 medium. At last, all the human colon carcinoma HT29 cells and supernatant in each well were collected and the radioactivity was calculated. The following formula was used to calculate the percentage of radioactivity within the cells of each well: cellular uptake rate (%) = radioactivity absorbed in each well/radioactivity added to each well.

Statistical analysis

The data were expressed as mean \pm SD and input into a computer for statistical analysis with SPSS 11 software. Differences among the groups were compared with paired *t*-test. *P* < 0.05 was considered statistically significant.

RESULTS

Labeling efficiency and radiochemical purity of labeled compounds

Analysis of labeling efficiency and radiochemical purity of the labeled compounds showed that the flow rate of ^{99m}Tc -HYNIC-ASON and ^{99m}Tc -MAG₃-ASON, $^{99m}\text{TcO}_4^-$, and deoxidized technetium, was 0.9-1.0, 0.6-0.7, and 0-0.1, respectively. The labeling efficiency and radiochemical purity of labeled compounds are

Table 1 Labeling efficacy and radiochemical purity of labeled compounds (mean \pm SD)

	Interval between binding and labeling (d)			Radioactivity of $^{99m}\text{TcO}_4^-$ (MBq)		
	15	30	60	370	740	1480
Labeling efficiency (%)						
Via HYNIC	57.36 \pm 3.69	62.13 \pm 4.25	62.87 \pm 3.04	58.74 \pm 5.32	62.86 \pm 4.27	63.28 \pm 3.38
Via NHS-MAG ₃	67.35 \pm 4.03	68.35 \pm 3.56	69.85 \pm 4.63	68.67 \pm 4.82	70.31 \pm 5.09	71.56 \pm 5.37
Radiochemical purity (%)						
^{99m}Tc -HYNIC-ASON	95.75 \pm 5.21	96.32 \pm 4.92	95.86 \pm 5.28	96.56 \pm 4.45	96.87 \pm 3.65	97.16 \pm 4.34
^{99m}Tc -MAG ₃ -ASON	96.43 \pm 4.69	95.67 \pm 5.17	96.39 \pm 4.78	96.35 \pm 6.12	95.86 \pm 4.67	96.54 \pm 5.65

ASON: Anti-sense oligonucleotide; HYNIC: Hydrazino nicotinamide derivative; NHS-MAG₃: N-hydroxysuccinimidyl S-acetylmercaptoacetyltriglycine.

Table 2 Radiochemical stability of labeled compounds (% mean \pm SD)

	Incubation time at room temperature (h)			Incubation time at 37°C (h)		
	1	2	4	1	2	4
^{99m}Tc -HYNIC-ASON	93.43 \pm 5.32	89.17 \pm 4.62	87.16 \pm 5.36	92.75 \pm 4.46	89.52 \pm 3.67	86.86 \pm 5.49
^{99m}Tc -MAG ₃ -ASON	97.26 \pm 6.02	96.68 \pm 5.54	96.39 \pm 4.68	95.86 \pm 5.69	95.47 \pm 4.07	94.79 \pm 5.34

Table 3 Biodistribution of labeled compounds in BALB/c mice (% ID/g) (mean \pm SD)

Tissue	0.5 h		1 h		2 h		4 h		Paired	t-test
	M	H	M	H	M	H	M	H		
Blood	1.12 \pm 0.76	6.21 \pm 1.03	2.38 \pm 0.63	6.56 \pm 1.11	1.14 \pm 0.42	3.58 \pm 1.21	1.10 \pm 0.09	2.83 \pm 0.54	$t = 4.347$	$P = 0.022$
Heart	0.62 \pm 0.31	2.13 \pm 0.45	0.58 \pm 0.07	1.64 \pm 0.34	0.32 \pm 0.05	1.16 \pm 0.12	0.18 \pm 0.08	0.81 \pm 0.13	$t = 5.362$	$P = 0.013$
Lungs	3.11 \pm 0.82	2.68 \pm 0.65	3.87 \pm 1.36	3.23 \pm 1.04	3.04 \pm 0.79	2.78 \pm 1.03	2.08 \pm 0.62	1.76 \pm 0.36	$t = -4.934$	$P = 0.016$
Liver	7.52 \pm 2.45	11.46 \pm 2.31	13.19 \pm 1.47	15.24 \pm 2.53	9.21 \pm 1.03	12.89 \pm 1.68	9.48 \pm 2.56	10.46 \pm 1.97	$t = 3.806$	$P = 0.032$
Kidneys	11.42 \pm 3.34	4.17 \pm 1.05	17.13 \pm 2.86	5.03 \pm 0.94	24.58 \pm 3.57	2.78 \pm 0.68	21.95 \pm 4.02	2.28 \pm 0.95	$t = -4.511$	$P = 0.020$
Spleen	2.71 \pm 1.62	4.87 \pm 2.36	5.65 \pm 0.93	6.08 \pm 1.93	5.35 \pm 0.26	4.08 \pm 1.54	4.84 \pm 1.33	5.21 \pm 2.04	$t = 0.603$	$P = 0.589$
Stomach	1.08 \pm 0.86	7.46 \pm 2.13	2.58 \pm 0.95	11.48 \pm 3.01	1.73 \pm 0.21	7.49 \pm 1.86	1.41 \pm 0.34	7.85 \pm 3.02	$t = 9.901$	$P = 0.002$
Intestines	0.53 \pm 0.31	1.26 \pm 0.31	0.86 \pm 0.14	2.68 \pm 0.95	1.23 \pm 0.19	6.44 \pm 2.13	1.96 \pm 0.53	7.84 \pm 2.34	$t = 2.706$	$P = 0.073$
Muscle	1.35 \pm 0.16	0.54 \pm 0.21	0.87 \pm 0.63	0.94 \pm 0.81	0.75 \pm 0.08	0.42 \pm 0.06	0.64 \pm 0.15	0.19 \pm 0.05	$t = -2.095$	$P = 0.127$

M: ^{99m}Tc -MAG₃-ASON; H: ^{99m}Tc -HYNIC-ASON. Statistical analysis of the same tissue distribution of radioactivity was made after the injection of ^{99m}Tc -HYNIC-ASON and ^{99m}Tc -MAG₃-ASON.

listed in Table 1. The labeling efficiency of ^{99m}Tc via NHS-MAG₃ was higher than that of ^{99m}Tc via HYNIC (for interval between binding and labeling: $t = 6.715$, $P = 0.021$; for radioactivity of $^{99m}\text{TcO}_4^-$: $t = 11.736$, $P = 0.007$). The radioactivity of $^{99m}\text{TcO}_4^-$ hardly influenced the labeling efficiency. The radiochemical purity of labeled compounds was higher than 95% and there was no statistical difference between the two methods (for interval between binding and labeling: $t = -0.444$, $P = 0.701$; for radioactivity of $^{99m}\text{TcO}_4^-$: $t = 2.656$, $P = 0.117$). Either interval between binding and labeling of HYNIC-ASON or that of MAG₃-ASON had almost no effect on the labeling efficiency and radiochemical purity of labeled compounds.

Radiochemical purity of labeled compounds

To assess the radiochemical purity, the labeled compounds were incubated at room temperature or at 37°C after diluted with an equal volume of fresh human serum (Table 2). The radiochemical purity of ^{99m}Tc -MAG₃-ASON was much higher than that of ^{99m}Tc -HYNIC-ASON (at room

temperature: $t = 5.616$, $P = 0.030$; at 37°C: $t = 5.616$, $P = 0.032$), while the radiochemical purity of ^{99m}Tc -MAG₃-ASON was less affected by incubation time than that of ^{99m}Tc -HYNIC-ASON.

Binding rate of rabbit plasma protein

The binding rate of rabbit serum protein for ^{99m}Tc -MAG₃-ASON or ^{99m}Tc -HYNIC-ASON was $11.17\% \pm 1.31\%$ and $71.06\% \pm 3.56\%$, respectively. The differences between them were statistically significant, and the former was lower than the latter ($t = 27.346$, $P < 0.0001$).

Biodistribution of ^{99m}Tc -MAG₃-ASON and ^{99m}Tc -HYNIC-ASON in BALB/c mice

The biodistributions of ^{99m}Tc -MAG₃-ASON and ^{99m}Tc -HYNIC-ASON in BALB/c mice are listed in Table 3. The distributions of ^{99m}Tc -MAG₃-ASON were significantly lower in blood, heart, liver and stomach than those of ^{99m}Tc -HYNIC-ASON ($P < 0.05$). The distributions of ^{99m}Tc -MAG₃-ASON were significantly higher in lungs and kidneys than those of ^{99m}Tc -HYNIC-ASON ($P < 0.05$).

Table 4 Human colon carcinoma HT29 cellular uptake of ^{99m}Tc-HYNIC-ASON and ^{99m}Tc-MAG₃-ASON (mean ± SD)

	10 min	20 min	40 min	60 min	120 min
^{99m} Tc-HYNIC-ASON (%)	0.43 ± 0.08	0.56 ± 0.21	0.93 ± 0.54	1.42 ± 0.64	1.67 ± 0.86
^{99m} Tc-MAG ₃ -ASON (%)	2.78 ± 0.81	5.64 ± 0.51	7.82 ± 2.53	13.63 ± 2.71	15.25 ± 3.13

There was no statistical difference in the distributions of ^{99m}Tc-MAG₃-ASON and ^{99m}Tc-HYNIC-ASON in spleen, intestines and muscle.

Cellular uptake of labeled compounds

Cellular uptake of labeled compounds in human colon carcinoma HT29 cells is listed in Table 4. The cellular uptake of ^{99m}Tc-MAG₃-ASON was significantly higher than that of ^{99m}Tc-HYNIC-ASON ($t = 3.770$, $P = 0.020$), which was 6.5, 10.1, 8.4, 9.5 and 9.1-folds higher than those of ^{99m}Tc-HYNIC-ASON at 10, 20, 40, 60 and 120 min after incubation.

DISCUSSION

In our studies, because expensive acetylsulfoacetic acid was not available, S-acetylthioglycolic acid N-hydroxysuccinimide ester (SATA) was synthesized as previously described^[5]. The synergistic coligand of tricine (N-tris-hydroxy-methyl-methylglycine) was applied to the synthesis of HYNIC, to achieve the high radioactivity of labeled compounds. During the synthesis of labeled compounds, isopropylol was used to crystallize the compounds instead of chromatographic column purification. Their synthesis was simple, efficient, economical, with a high yield (75%-80%) and little environmental pollution. Nuclear magnetic resonance of labeled compounds was performed as previously described^[5-7]. Both Sep-Pak C18 reversed-phase column and Sephadex G25 column could be used to purify the radiolabeled ASON. Both labeling methods can achieve a high radiochemical purity of over 95%.

Stability can be obtained by methylation, amination or sulfonation of the phosphorus atoms in ASON, making it not recognized and degraded by nucleic acid enzyme^[8]. In the present study, we modified the ASON by replacing the hydroxyl group in the phosphoric acid branch of ASON with a sulphur atom and attaching an amid to the 5' terminal of ASON. Labeled compounds were observed for four hours to detect the stability of ASON labeled with ^{99m}Tc *via* HYNIC or NHS-MAG₃. Only 1-2 covalent bonds were formed between a molecule of HYNIC-ASON and a technetium atom. However, it was reported that 4-5 covalent bonds can form between MAG₃-ASON and technetium^[9,10], which may be the reason for a greater stability of ^{99m}Tc-MAG₃-ASON than that of ^{99m}Tc-HYNIC-ASON. During labeling, since the mercapto group of ASON-MAG₃ is protected by acetyl group, excessive SnCl₂ is needed to hydrolyze the protection group of ASON-MAG₃^[11], which may be the reason for a greater labeling efficiency of ^{99m}Tc *via* NHS-MAG₃ than *via* HYNIC.

The binding rate of ^{99m}Tc-MAG₃-ASON to rabbit serum protein was significantly lower than that of ^{99m}Tc-HYNIC-ASON in our study, suggesting that the distributions of ^{99m}Tc-MAG₃-ASON are significantly lower in blood, heart and liver of BALB/c mice. The distributions of ^{99m}Tc-MAG₃-ASON were much lower in stomach than those of ^{99m}Tc-HYNIC-ASON, suggesting that ^{99m}Tc-MAG₃-ASON has a greater *in vivo* ability than ^{99m}Tc-HYNIC-ASON. The distributions of ^{99m}Tc-MAG₃-ASON were much higher in kidneys than those of ^{99m}Tc-HYNIC-ASON, which may be related to the metabolism of MAG₃-ASON in kidneys.

Cellular targeting uptake of ASON can be improved by receptor-mediated mechanisms^[12-14]. The conjugation of vasoactive intestinal peptide (VIP)-ASON is very helpful for ¹²⁵I-ASON to selectively bind to HT29 tumor cells by VIP receptors. For such tumor cells that highly express VIP receptors, tumor cellular uptake of VIP-¹²⁵I-ASON is significantly higher than that of ¹²⁵I-ASON un-conjugated to VIP^[12]. The c-myc ASON complex entered human melanoma cells (M14) by folacin receptors on tumor cell surface, brings about a greater cellular uptake than that of free-ASON, and inhibits tumor growth by lowering c-myc cancer protein expression^[13]. As we know, the c-myc oncogene and transferrin receptors are highly expressed in HL-60 and LoVo Dx cells, the addition of transferrin-polylysine-c-myc ASON complex would cause more tumor cell deaths than free c-myc ASON^[14]. However, receptor mediation was not used in our study. Why HT29 cellular uptake of ^{99m}Tc-MAG₃-ASON is higher than that of ^{99m}Tc-HYNIC-ASON is unclear, which is possibly related to the greater stability of ^{99m}Tc-MAG₃-ASON, and needs further study.

COMMENTS

Background

Anti-sense oligonucleotide (ASON) is used to bind to deoxyribonucleic acid (DNA) translation or transcription and interfere with the expression of oncogene. However, ASON is not sufficient to inhibit tumor growth. In order to enhance anti-tumor effect of ASON, we labeled ASON with technetium-99m *via* N-hydroxysuccinimidyl S-acetylmercaptoacetyltriglycine (NHS-MAG₃) and hydrazinonictinamide derivative (HYNIC).

Research frontiers

Many proteins such as monoclonal antibody, polypeptide, ligand, can be labeled with radionuclides, such as ¹²⁵I, ¹³¹I, ³²P, ³⁵S, ^{99m}Tc, ¹⁸⁸Re, ¹⁸⁶Re, ⁹⁰Y. We are trying to label oncolytic virus with radionuclide, in order to achieve a synergistic anticancer effect.

Innovations and breakthroughs

In this study, we compared the radiochemical behaviors and biological properties of anti-sense oligonucleotide (ASON) labeled with technetium-99m *via* N-hydroxysuccinimidyl S-acetylmercaptoacetyltriglycine (NHS-MAG₃) and

hydrazinonictinamide derivative (HYNIC) and found that ^{99m}Tc -MAG₃-ASON showed superior radiochemical behaviors and biological properties than ^{99m}Tc -HYNIC-ASON.

Applications

^{99m}Tc -MAG₃-ASON showed superior radiochemical behaviors and biological properties than ^{99m}Tc -HYNIC-ASON, suggesting that it can be used as a potential radiopharmaceutical agent for *in vivo* application.

Peer review

In this study, the authors analyzed and compared the radiochemical behaviors and biological properties of anti-sense oligonucleotide (ASON) labeled with technetium-99m via NHS-MAG₃ and HYNIC. The rationale of the study is clearly expressed and the experiments appear to be carefully conducted.

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Effects of simulated carbon dioxide and helium pneumoperitoneum on proliferation and apoptosis of gastric cancer cells

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Abstract

AIM: To investigate the effects of carbon dioxide (CO₂) and helium insufflation administered at different pressures on the growth and apoptosis of cultured human gastric cancer cells.

METHODS: The gastric cancer cells MKN-45 were exposed to a CO₂ and helium environment maintained at different pressures (0, 5, 10 and 15 mmHg). The cells were exposed to simulated pneumoperitoneum environment for 4 h, and pH of the culture media was measured after it was moved to normal conditions for 0, 2, 4, 6 and 8 h. Proliferation viability of MKN-45 was examined by 3-[4,5Dimethylthiazol-2-yl],5-diphenyltetrazolium bromide or triazolyl blue (MTT) assay after it was moved to normal conditions. Apoptotic ratio was measured by Annexin V-FITC/PI double labelled staining.

RESULTS: The pH of media was acid and recovered to normal after 4 h in the CO₂ group while it was basic in the helium group. There was no difference between CO₂ groups (under 10 mmHg) and control group ($P > 0.05$) in the proliferative viability of the cells. The cultured cells exposed to 15 mmHg CO₂ environment grew more slowly than control group from 4 to 7 d ($P < 0.01$) while there was no difference from 1 to 3 d ($P > 0.05$). The proliferative viability in helium group was not obviously different from the control group ($P > 0.05$). The

apoptotic ratio of the cultured cells was markedly higher than that of the control group ($P < 0.01$) at 10 and 15 mmHg CO₂ insufflation pressure. In helium group, the apoptotic ratio was not obviously different from the control group ($P > 0.05$).

CONCLUSION: There is no obvious effect in the proliferation and apoptosis of MKN-45 cells under 10 mmHg CO₂ insufflation pressure and helium in any pressure. Fifteen mmHg CO₂ insufflation pressure can inhibit the proliferation of the cells and improve apoptosis.

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Key words: Pneumoperitoneum; Gastric cancer cells; Proliferation; Apoptosis

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INTRODUCTION

Minimally invasive techniques are increasingly applied in abdominal surgeries^[1,2]. In recent years, numerous authors reported an acceptable feasibility of minimally invasive techniques for biopsy and resection of various malignant tumors^[3,4]. However, laparoscopic resection for intra-peritoneal malignancies remains controversial. One of the reasons is the concern whether carbon dioxide (CO₂) pneumoperitoneum can improve cancer cells' growth^[5,6]. There is an ongoing debate about the deleterious effects of CO₂ on tumor cell behavior. Some authors showed an increase in cell proliferation and tumor growth^[7] and others found beneficial effects of CO₂ exposition *in vitro* and in animal studies^[8,9].

It is well known that intracellular and extracellular pH in the peritoneum is affected by CO₂ insufflation. And some authors reported that pH in peritoneal cavity may be an important regulator of cell functions, such as adenosine

triphosphate (ATP) production, cell proliferation, and apoptosis^[10]. Apart from the acid of peritoneal cavity, whether the direct insult of insufflation pressure could affect the growth of tumor cells is unclear.

Therefore, we focused on the different gases and pressures in simulated pneumoperitoneum, and investigated the proliferative viability of gastric cancer cells and apoptotic ratio *in vitro*.

MATERIALS AND METHODS

Cell culture

Human gastric cancer cells (MKN-45; personal gift of Professor F Daiming, Fourth Military Medical University) were cultured in RPMI-1640 (HyClone, USA) culture medium supplemented with 100 g/L fetal bovine serum, penicillin G 100 IU/mL and streptomycin sulfate 100 µg/mL.

Pneumoperitoneum model *in vitro*

To simulate the environment produced during laparoscopic surgery, we designed an *in vitro* pneumoperitoneum according to Ridgway's method^[11]. We used 100% CO₂ or 100% helium as the insufflation gas-displacement model. Sub-confluent MKN-45 cells which had been plated on 6 cm Petri dishes were placed into modified desiccating chambers. CO₂ or helium insufflation was affected by the connection of a standard surgical insufflator (Stryker, USA) to the chamber. Cells were exposed to a continual pneumoperitoneum for 4 h at 0, 5, 10 and 15 mmHg at 37°C. The pH of the media was examined using an arterial blood gas analyzer (Radiometer ABL 505, Denmark). After the cells were exposed to CO₂ or helium for 4 h, the media was changed and the cells were allowed to grow for 24 h before 3-[4,5Dimethylthiazol-2-yl],5-diphenyltetrazolium bromide or triazolyl blue (MTT) assay or flow cytometry.

Cell viability

Cell growth was determined with a spectrophotometric assay^[12]. This water-soluble tetrazolium salt was cleaved by the mitochondrion of living cells to an insoluble purple formazan. Optical density readings were measured at 490 nm.

Percentage of apoptotic cells

The percentage of apoptotic cells was determined by FITC-labeled Annexin V and PI double staining flow cytometry.

The cell growth and apoptosis for each group were compared with those of the control group using one-way analysis of variance (ANOVA). *P* values less than 0.05 were considered significant.

RESULTS

Influence of pneumoperitoneum on pH of media

The pH of media in CO₂ and helium group is shown in Tables 1 and 2. When the pressure of CO₂ pneumoperitoneum was 15 mmHg, the pH of media was 6.18. It became normal after 4 h when moved to normal

Table 1 Changes of culture media pH in CO₂ groups (*n* = 4) (mean ± SD)

Groups	Time (h)				
	0	2	4	6	8
Control	7.20 ± 0.02	7.18 ± 0.02	7.15 ± 0.01	7.16 ± 0.03	7.20 ± 0.02
0 mmHg	7.13 ± 0.04 ^b	7.15 ± 0.03	7.15 ± 0.03	7.19 ± 0.01	7.23 ± 0.04
5 mmHg	7.00 ± 0.05 ^b	7.13 ± 0.03	7.22 ± 0.02	7.22 ± 0.02	7.24 ± 0.02
10 mmHg	6.77 ± 0.03 ^b	6.95 ± 0.05 ^b	7.16 ± 0.03	7.22 ± 0.01	7.21 ± 0.01
15 mmHg	6.18 ± 0.02 ^b	6.91 ± 0.02 ^b	7.08 ± 0.04	7.20 ± 0.02	7.22 ± 0.01

^b*P* < 0.01 vs control group.

Table 2 Changes of culture media pH in helium groups (*n* = 4) (mean ± SD)

Groups	Time (h)				
	0	2	4	6	8
Control	7.20 ± 0.01	7.18 ± 0.02	7.15 ± 0.01	7.17 ± 0.03	7.20 ± 0.02
0 mmHg	7.42 ± 0.02 ^b	7.23 ± 0.03	7.18 ± 0.01	7.15 ± 0.01	7.16 ± 0.03
5 mmHg	7.53 ± 0.03 ^b	7.28 ± 0.02 ^b	7.21 ± 0.02 ^a	7.19 ± 0.03	7.15 ± 0.01
10 mmHg	7.82 ± 0.02 ^b	7.31 ± 0.01 ^b	7.23 ± 0.02 ^b	7.20 ± 0.05	7.15 ± 0.02
15 mmHg	8.19 ± 0.04 ^b	7.96 ± 0.03 ^b	7.33 ± 0.05 ^b	7.22 ± 0.01	7.19 ± 0.02

^a*P* < 0.05 vs control group, ^b*P* < 0.01 vs control group.

cultured environment. In the helium group, the pH of the media was 8.12 when the pressure was 15 mmHg. Six hours later, it dropped to 7.18 when it was moved to normal cultured environment (Tables 1 and 2).

MTT assay

According to MTT chromometry, the proliferative viability of MKN-45 cells was significantly decreased from d 4 to d 7 after it was exposed to simulated CO₂ pneumoperitoneum at 15 mmHg. When the pressure was under 10 mmHg, the cells' proliferative viability was not obviously different from the control group (*P* > 0.05). In the helium group, there was no difference between various pressures and control group (*P* > 0.05), even at 15 mmHg (Tables 3 and 4).

Percentage of apoptotic cells

The percentage of apoptotic cells in 10 and 15 mmHg CO₂ groups was significantly higher than control group (*P* < 0.01). In the helium group, there was no significant difference in the percentage of apoptotic cells under different pressures (*P* > 0.05). Even the pressure was 15 mmHg, there was no significant difference from the control group (*P* > 0.05) (Table 5).

DISCUSSION

Several prospective, randomized studies on laparoscopically assisted surgeries for early gastric cancer have demonstrated that the 5-year survival of patients with laparoscopically assisted radical resection of gastric carcinomas was similar to or even higher than that of open surgery^[13]. Since March 2004, we have performed 304 cases of laparoscopically

Table 3 Changes of MKN-45 proliferative viability in CO₂ groups (OD, mean \pm SD)

Groups	Time (d)						
	1	2	3	4	5	6	7
Control	0.31 \pm 0.04	0.41 \pm 0.02	0.53 \pm 0.09	1.38 \pm 0.04	1.81 \pm 0.09	2.33 \pm 0.04	2.33 \pm 0.06
0 mmHg	0.28 \pm 0.06	0.41 \pm 0.04	0.56 \pm 0.06	1.37 \pm 0.07	1.58 \pm 0.02	2.38 \pm 0.06	2.39 \pm 0.08
5 mmHg	0.31 \pm 0.05	0.35 \pm 0.05	0.56 \pm 0.04	1.34 \pm 0.04	1.59 \pm 0.07	2.50 \pm 0.07	2.32 \pm 0.04
10 mmHg	0.29 \pm 0.02	0.36 \pm 0.04	0.53 \pm 0.05	1.27 \pm 0.05	1.57 \pm 0.14	2.54 \pm 0.10	2.40 \pm 0.03
15 mmHg	0.32 \pm 0.03	0.39 \pm 0.05	0.47 \pm 0.05	0.68 \pm 0.04 ^b	0.80 \pm 0.04 ^b	1.16 \pm 0.08 ^b	1.42 \pm 0.02 ^b

^b*P* < 0.01 vs control group; OD: Optical density.Table 4 Changes of MKN-45 proliferative viability in helium groups (OD, mean \pm SD)

Groups	Time (d)						
	1	2	3	4	5	6	7
Control	0.29 \pm 0.04	0.36 \pm 0.04	0.58 \pm 0.03	1.22 \pm 0.05	1.83 \pm 0.03	2.21 \pm 0.04	2.62 \pm 0.04
0 mmHg	0.30 \pm 0.02	0.39 \pm 0.02	0.60 \pm 0.04	1.23 \pm 0.06	1.86 \pm 0.06	2.37 \pm 0.05	2.64 \pm 0.05
5 mmHg	0.30 \pm 0.02	0.38 \pm 0.03	0.58 \pm 0.03	1.27 \pm 0.05	1.80 \pm 0.08	2.40 \pm 0.33	2.75 \pm 0.12
10 mmHg	0.31 \pm 0.03	0.41 \pm 0.03	0.57 \pm 0.05	1.25 \pm 0.06	1.78 \pm 0.04	2.49 \pm 0.26	2.71 \pm 0.18
15 mmHg	0.33 \pm 0.02	0.39 \pm 0.04	0.54 \pm 0.09	1.24 \pm 0.23	1.81 \pm 0.09	2.31 \pm 0.20	2.73 \pm 0.11

OD: Optical density.

Table 5 Changes of MKN-45 apoptosis ratio in CO₂ and helium groups (% mean \pm SD)

Groups	Control	0 mmHg	5 mmHg	10 mmHg	15 mmHg	F
CO ₂	0.21 \pm 0.02	0.19 \pm 0.04	0.29 \pm 0.05	9.20 \pm 0.44 ^a	11.60 \pm 0.95 ^a	430.09
He	0.28 \pm 0.04	0.27 \pm 0.04	0.31 \pm 0.08	0.35 \pm 0.11	0.37 \pm 0.05	0.99

^a*P* < 0.05 vs control group; CO₂: Carbon dioxide; He: Helium.

assisted gastrectomy, 236 of the cases were advanced gastric cancer. We found no obvious difference between excising tumor with tumor-free margin and dissecting lymph nodes radically^[14,15]. However, laparoscopic resection for abdominal malignancy remains controversial, especially for advanced gastric cancer. Among the reasons for this is the concern whether CO₂ pneumoperitoneum can improve port-site metastasis, peritoneal dissemination and recurrence^[5,16,17].

The results of experimental studies on the behavior of tumor cells exposed to CO₂ are not conclusive. Numerous authors confirmed a CO₂ associated increase of tumor growth and invasiveness of various cell lines derived from colon carcinoma, adenocarcinoma, and other tumors using animal models^[18-20]. However, other studies showed that CO₂ pneumoperitoneum could increase cell necrosis and decrease proliferation^[8,21]. Our data indicated that the exposure to CO₂ decreased the mitochondrial activity of MKN-45 cells, especially in a higher pressure environment (15 mmHg). We noticed this change when it was moved to normal culture environment for 4 h. The percentage of apoptotic cells increased in CO₂ pneumoperitoneum (10 and 15 mmHg group). This phenomenon was also investigated in human ovarian cancer cell lines HO8901, SKVO3^[22] and other tumor cells^[23,24].

Helium has been suggested for alternative use for

pneumoperitoneum to prevent CO₂ effects such as local acidosis and systemic hypercapnia^[25]. In addition, a beneficial effect of helium versus CO₂ on the growth of rat mammary adenocarcinoma cells was shown *in vitro*^[26]. In our experiments, we observed no obvious difference between helium group and control group, even at 15 mmHg pressure. This proved that the increase of cell apoptotic ratio in CO₂ pneumoperitoneum might not only depend on insufflated pressure.

Kos *et al* showed that intracellular and extracellular acidification associated with CO₂ resulted in an attenuation of cytokine release and cell activity in macrophages^[27]. Takiguchi *et al* believed CO₂ pneumoperitoneum had no effect on cancer cells' proliferative ratio but had a toxic effect on cancer cells^[18].

Our current experiments confirmed that the extracellular pH differed significantly between CO₂ and helium exposure and it decreased very sharply at the insufflated pressure. Wildbrett *et al* reported that intracellular and extracellular pH and calcium level were altered with CO₂ pneumoperitoneum^[10]. pH and calcium are important regulators of cell functions such as ATP production, cell cycle, intracellular signaling and apoptosis^[28,29]. It is likely that all these changes influence the favorability of tumor-cell implantation at the time of laparoscopic surgery.

West *et al* excluded hypoxia as a cause of alteration of cell functions by exposing cells to 20% and 80% CO₂^[30]. In our experiments, exposition to both 100% CO₂ and 100% helium may cause hypoxia, but the impact on MKN-45 gastric cancer cells was significantly different. Only CO₂ reduced cell activity, which made no hypoxic effects. The direct effects of CO₂ demonstrated by Takiguchi *et al* on human colon cancer cells *in vitro*^[18] remain to be confirmed for gastric cancer cells.

CO₂ pneumoperitoneum resulted in severe peritoneal

acidosis, and peritoneal acidosis may play a role in changing tumor cells' implantation during laparoscopic oncologic surgery. The role of peritoneal microenvironment in tumor-cell growth awaits further studies. More studies in the area could enable us to find the safest approach to laparoscopic oncologic surgery.

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COMMENTS

Background

Laparoscopic surgery in oncologic patients is increasingly adopted as an alternative to conventional surgical procedures, both for diagnosis and resection. However, some experimental and clinical studies have suggested that the CO₂ pneumoperitoneum influences the development of intra-abdominal tumor dissemination and port site metastases. Numerous authors confirmed a CO₂ associated increase of tumor growth and invasiveness of various cell lines derived from colon carcinoma, adenocarcinoma, and other tumors. However, other studies showed beneficial effects of CO₂, such as increased cell necrosis and decreased proliferation.

Research frontiers

The effects of laparoscopic environment on tumor cell biology, including the kind of gas and the pressure of pneumoperitoneum.

Innovations and breakthroughs

The results of experimental studies on the behavior of tumor cells exposed to CO₂ are not conclusive. In this study, the authors elaborately and clearly demonstrate that it is the CO₂ gas and not the pressure or the hypoxia that inhibits the growth of the cancer cells and increases apoptosis.

Applications

Laparoscopic resection for intra-abdominal malignancies remains controversial, especially for advanced gastric cancer. One of the reasons is the concern whether CO₂ pneumoperitoneum can improve port-site metastasis, peritoneal dissemination and recurrence. This research on CO₂ pneumoperitoneum could improve the application of CO₂ as the insufflation gas in laparoscopic surgery.

Terminology

Helium insufflation: The act of blowing helium into any body cavity for experimental, diagnostic, or therapeutic purposes. CO₂ pneumoperitoneum: The presence of CO₂ in the peritoneal cavity. It may occur spontaneously or be deliberately introduced as an aid to operate.

Peer review

This is a good study. As far as the *in vitro* effects of gases and pressure on cancer cell growth and apoptosis is concerned, one can find studies reporting exactly contradictory findings. The authors elaborately and clearly demonstrate that it is the CO₂ gas and not the pressure or the hypoxia that inhibits the growth of the cancer cells and increases apoptosis.

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RAPID COMMUNICATION

Dynamic changes of IL-2/IL-10, sFas and expression of Fas in intestinal mucosa in rats with acute necrotizing pancreatitis

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Abstract

AIM: To investigate dynamic changes of serum IL-2, IL-10, IL-2/IL-10 and sFas in rats with acute necrotizing pancreatitis. To explore the expression of Fas in intestinal mucosa of rats with acute necrotizing pancreatitis (ANP).

METHODS: A total of 64 Sprague-Dawley (SD) rats were randomly divided into two groups: normal control group (C group), ANP group (P group). An ANP model was induced by injection of 50 g/L sodium taurocholate under the pancreatic membrane. Normal control group received isovolumetric injection of 9 g/L physiological saline solution using the same method. The blood samples of the rats in each group were obtained via superior mesenteric vein to measure levels of IL-2, IL-10, sFas and calculate the value of IL-2/IL-10. The levels of IL-2, IL-10 and sFas were determined by ELISA. The severity of intestinal mucosal injury was evaluated by pathologic score. The expression of Fas in intestinal mucosal tissue was determined by immunohistochemistry staining.

RESULTS: Levels of serum IL-2 were significantly higher in P group than those of C group (2.79 ± 0.51 vs 3.53 ± 0.62 , 2.93 ± 0.89 vs 4.35 ± 1.11 , 4.81 ± 1.23 vs 6.94 ± 1.55 and 3.41 ± 0.72 vs 4.80 ± 1.10 , respectively, $P < 0.01$, for all) and its reached peak at 6 h. Levels of serum IL-10 were significantly higher in P group than those of C group at 6 h and 12 h (54.61 ± 15.81 vs 47.34 ± 14.62 , 141.15 ± 40.21 vs 156.12 ± 43.10 , 89.18 ± 32.52 vs 494.98 ± 11.23 and 77.15 ± 22.60 vs 93.28 ± 25.81 , respectively, $P < 0.01$, for all). The values of IL-2/IL-10 were higher significantly in P group than those of C group at 0.5 h and 2 h (0.05 ± 0.01 vs 0.07 ± 0.02 and 0.02 ± 0.01 vs

0.03 ± 0.01 , respectively, $P < 0.01$, for all), and it were significantly lower than those of C group at 6 h (0.05 ± 0.02 vs 0.01 ± 0.01 , $P < 0.01$) and returned to the control level at 12 h (0.04 ± 0.01 vs 0.05 ± 0.02 , $P > 0.05$). In sFas assay, there was no significant difference between P group and C group (3.16 ± 0.75 vs 3.31 ± 0.80 , 4.05 ± 1.08 vs 4.32 ± 1.11 , 5.93 ± 1.52 vs 5.41 ± 1.47 and 4.62 ± 1.23 vs 4.44 ± 1.16 , respectively, $P > 0.05$, for all). Comparison of P group and C group, the pathological changes were aggravated significantly in P group. Immunohistochemistry staining show the expression of Fas was absent in normal intestinal tissues, however, it gradually increased after induction of pancreatitis in intestinal tissue, then reached their peaks at 12 h.

CONCLUSION: Fas were involved in the pathogenesis of pancreatitis associated intestinal injury. The mechanisms of Fas may be associated to Fas mediated T helper cell apoptosis.

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Key words: Acute necrotizing pancreatitis; Fas; Intestinal mucosal injury; T helper cell

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INTRODUCTION

Acute pancreatitis (AP) is sudden inflammation of the pancreas that may be mild or life threatening but that usually subsides. Although usually self-limiting, 10% to 20% of afflicted patients will progress to acute necrotizing pancreatitis (ANP)^[1,2]. The mortality rate among patients with ANP may approach 30% when they progress to multiple organ failure (MOF)^[3]. It is generally accepted that AP is often complicated by intestinal injury. Failure of intestinal barrier function often occurs in this condition, resulting in the increased intestinal permeability. It is clear that increased intestinal permeability and bacteria with

or without endotoxin translocation plays a key role in the development of severe complications such as systemic inflammatory response syndrome (SIRS), sepsis, multiple organ dysfunction syndrome (MODS) and MOF^[4-7]. However, its pathogenesis remains unclear.

The Fas system was originally characterized as a key mechanism for inducing apoptosis in immune cells, but later it transpired to be very common in various tissues such as liver, ovary, kidney, and testis, especially under I/R conditions. Apoptosis is a teleologically beneficial form of cell death in AP. However, little is known about how the induction of apoptosis reduces the severity of AP^[8]. Recent research has demonstrated that a Th1 to Th2 immune deviation is beneficial to ANP. The antigen-induced deletion of Th is often accompanied by an imbalance in Th1 and Th2. The two most polarized patterns of cytokine production, Th1 (characterized by production of IL-2, IFN- γ , TNF- α , and TNF- β) and Th2 (characterized by production of IL-4, 5, 6, 10, and -13) were reported^[9].

In the present study, we performed immunohistochemistry staining of apoptosis-related protein Fas and investigated dynamic changes of serum IL-2, IL-10, IL-2/IL-10 and sFas in rats with ANP.

MATERIALS AND METHODS

Animals

Adult Sprague-Dawley rats of both sexes weighing 250-300 g were provided by the Laboratory Animal Center of Jiangsu University. The animals were fed with standard rat chow and water *ad libitum*. The rats were allowed to acclimatize to our laboratory conditions for 1 wk and then subjected to mesh stainless-steel cages at a constant temperature ($21 \pm 1^\circ$) in a 12 h day/night cycle. The animals were fasted for 12 h before the experiments but had free access to water. Animal care and experimental procedure were performed in accordance with the guidelines for Animal Experimentation of Jiangsu University with the approval of the Institutional Animal Care and Use Committee.

Experimental design

The animals were randomly divided into: control group (C group), ANP group (P group) with 32 rats in each group. Each group was further divided into 0.5, 2, 6 and 12 h subgroups, respectively. The mortality in the present series was not calculated. The rats were anesthetized with an intraperitoneal injection of sodium pentobarbital (50 mg/kg body weight). The rats were infused with sodium taurocholate (4 mL/kg, Na-Tc, Sigma) the pancreatic membrane to induce ANP model as previously described^[10]. After 30 min, pancreatic edema and dotted bleeding occurred. Normal control group received isovolumetric injection of 9 g/L physiological saline solution using the same method. Animals in each group were sacrificed at 0.5, 2, 6 and 12 h after infusion for further examination. Part of distal ileum and pancreas were removed immediately and fixed in paraformaldehyde solution for 12-24 h and paraffin-embedded for routine histopathologic analysis. The histopathologists were blinded to routine histopathologic analysis.

Analysis of Th1/Th2 cytokines and sFas

The blood of rats in each group was obtained via superior mesenteric vein for determination of serum IL-2, IL-10 and sFas levels at 0.5, 2, 6 and 12 h after infusion. Serum levels of IL-2, IL-10 and sFas were measured by double antibody sandwich ELISA according to the manufacturer's protocol (Shanghai Senxiong Technology Enterprise Co., Ltd.). The optical density of each well was determined within 30 min using a microplate reader (492 nm).

Pathological examination

The whole pancreas and parts of distal ileum were obtained and promptly fixed in 40 g/L phosphate-buffered formaldehyde for further studies. Paraffin-embedded tissue sections (5 μ m thick) were stained with hematoxylin and eosin. Mucosal damage was assessed according to the standard scale of Chiu *et al.*^[11]. Grading was performed and classified as 0 = normal mucosa; 1 = development of subepithelial space at the tip of the villus; 2 = extension of the space with epithelial lifting; 3 = massive epithelial lifting; 4 = denuded villi; 5 = disintegration of the lamina propria.

Immunohistochemistry

After embedding in paraffin, sections 5 μ m in thickness were immersed twice into xylene for 5 min each, followed by immersion twice for 3 min each in 100% ethanol and then 95% ethanol. Slides were rinsed for 30 sec using deionized water and then immersed twice in deionized water for 5 min. To detect Fas expression, heat-induced Ag retrieval was performed using 0.01 mol/L citrate buffer (pH 6.2) and 10 min slide immersion into 95°C waterbath. Immunoenzyme double staining of intestinal tissue was performed using DAKO EnVision Doublestain System. The sections were then counterstained using hematoxylin before study.

Statistical analysis

All data were analyzed with the SPSS 11.0 software. The results were expressed in mean \pm SD except for data on the grading of intestinal mucosal lesions. Differences of grading of intestinal mucosal lesions were determined using the non-parametric Mann-Whitney test. Statistical analysis was performed with post-hoc test. $P < 0.05$ was considered statistically significant.

RESULTS

Serum IL-2 level

At 0.5 h after injection of 50 g/L sodium taurocholate, serum IL-2 level in the samples from mesentery vein in P group were higher than those in C group. From 0.5 h, there was a significant difference between P group and C group ($P < 0.01$, Table 1).

Serum IL-10 level

Upon stimulation, serum IL-10 level was significantly increased in the P group as compared with that of C group at 6 and 12 h ($P < 0.01$, Table 1). There was no significant difference between P group and C group at 0.5 and 2 h.

Table 1 IL-2 and IL-10 level in each group (mean \pm SD, $n = 8$) (pg/mL)

Group		0.5 h	2 h	6 h	12 h
IL-2	C	2.79 \pm 0.51	2.93 \pm 0.89	4.81 \pm 1.23	3.41 \pm 0.72
	P	3.53 \pm 0.62 ^b	4.35 \pm 1.11 ^b	6.94 \pm 1.55 ^b	4.80 \pm 1.10 ^b
IL-10	C	54.61 \pm 15.81	141.15 \pm 40.21	89.18 \pm 32.52	77.15 \pm 22.60
	P	47.34 \pm 14.62	156.12 \pm 43.10	494.98 \pm 11.23 ^b	93.28 \pm 25.81 ^b

^b $P < 0.01$ vs control group.**Table 2 Serum IL-2/IL-10 AND Serum sFas in each group (mean \pm SD, $n = 8$)**

Group		0.5 h	2 h	6 h	12 h
IL-2/IL-10	C	0.05 \pm 0.01	0.02 \pm 0.01	0.05 \pm 0.02	0.04 \pm 0.01
	P	0.07 \pm 0.02 ^b	0.03 \pm 0.01 ^b	0.01 \pm 0.01 ^b	0.05 \pm 0.02
sFas (pg/mL)	C	3.16 \pm 0.75	4.05 \pm 1.08	5.93 \pm 1.52	4.62 \pm 1.23
	P	3.31 \pm 0.80 ^a	4.32 \pm 1.11 ^a	5.41 \pm 1.47 ^a	4.44 \pm 1.16 ^a

^a $P > 0.05$ vs control group, ^b $P < 0.01$ vs control group.**Table 3 Intestinal mucosal injury in each group ($n = 8$)**

Group	0.5 h						2 h						6 h						12 h					
	0	1	2	3	4	5	0	1	2	3	4	5	0	1	2	3	4	5	0	1	2	3	4	5
C	7	1	0	0	0	0	6	2	0	0	0	0	7	1	0	0	0	0	6	2	0	0	0	0
P	0	2	5	1	0	0 ^b	0	0	2	4	2	0 ^b	0	0	0	1	4	3 ^b	0	0	0	0	3	5 ^b

^b $P < 0.01$ vs C group.

Serum IL-2/IL-10

As shown in Table 2, the values of IL-2/IL-10 were higher significantly in P group than those of C group at 0.5 h and 2 h, and it were significantly lower than those of C group at 6 h ($P < 0.01$) and returned to the control level at 12 h ($P > 0.05$) (Table 2).

Serum sFas level

In serum sFas assay as illustrated Table 2, there was no significant difference between P group and C group. sFas levels were moderate at 0.5 h, peaked at 6 h and decreased at 12 h.

Pathologic examination of pancreas and intestinal mucosa

After induction of ANP model, pancreas showed mild edema and congestion. 2 h after introduction of the model, typical pathologic changes were found in P group, such as a large number of inflammatory cells, necrosis of adjacent fat tissues, interstitial edema, parenchyma hemorrhage and necrosis, large amount of ascites. The degree of intestinal pathological injury is shown in Table 3. The grades of P group were significantly higher those of control group ($P < 0.01$).

Immunohistochemistry

We detected the expression of Fas on intestinal tissue with immunocytochemical technique. Immunohistochemistry staining showed Fas expression in intestinal tissue was absent in normal intestinal tissue, Fas expression in intestinal tissue gradually increased 0.5 h after induction of pancreatitis, and then reached a peak at 12 h (Figure 1).

DISCUSSION

The Fas system is a widely recognized apoptotic signal transduction pathway in which a ligand-receptor interaction triggers the cell death pathway^[12]. The Fas system has been implicated in the control of the immune response and inflammation, the response to infection and

death of parenchymal cells in several organs^[13-15], which is involved in maintaining homeostasis in various systems, including maintenance of peripheral T cell^[16]. Recent research has demonstrated that a Th1 to Th2 immune shift is beneficial to mucosal immunity^[17,18]. A key component of the mucosal immune defense against pathogens is mediated by CD4+ T lymphocytes that can differentiate into functionally distinct subsets^[19]. Whereas T-helper 1 (Th1) cells secrete the cytokines IL-2, IFN- γ , TNF- α , and TNF- β , Th2 cells secrete IL-4, 5, 6, 10, and 13. In the current study, we used IL-2 levels as a marker of Th1 response and IL-10 as a marker of Th2 response. In this study, the role of the Fas-mediated cell death pathway in intestinal mucosal injury models was assessed and dynamic changes of serum IL-2, IL-10, IL-2/IL-10 and sFas in rats with ANP were also investigated.

According to the result of immunohistochemistry, Fas had a lower expression in intestinal tissue in the P group at 0.5 h and higher expression after 2, 6 and 12 h. In the C groups, Fas was not detected in any part of intestinal tissue. Previous study has shown that IL-10 is a kind of important anti-inflammatory cytokine and plays a role of self-defense mechanism, limiting the intensity of inflammatory process^[20-23]. However, the effect of IL-10 level in the course of acute pancreatitis is still not clear^[24-26]. IL-10 is a powerful Th2 cell cytokine produced by lymphoid cells. A marked activation of immune system may be observed in patients with AP, being balanced between pro- and anti-inflammatory cytokines in patients with mild but not severe AP. A reduced functional reserve for the synthesis of IL-10 may be observed in patients with severe AP, which might lead to a worst prognosis^[27-30].

From the results of this study, it was found that the release of IL-10 was significantly increased in the P group as compared with that of C group at 6 and 12 h. Serum IL-2 level in the P group were higher than those in C group. From 0.5 h, there was a significant difference between P group and C group. The IL-2/IL-10 ratio was significantly increased in the P group as compared with that of C group

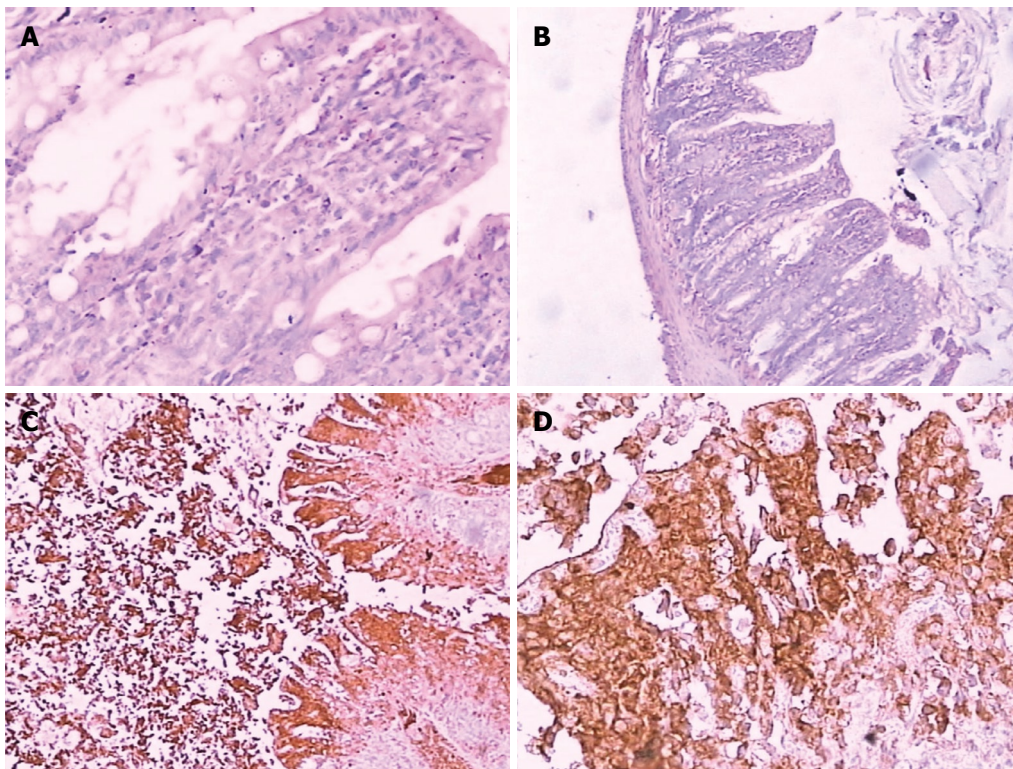


Figure 1 Morphological changes intestinal mucosa after induction of ANP. **A:** Intestinal section with normal mucosa (ANP 0.5 h); **B:** Disintegration of the lamina propria (ANP 6 h); **C:** Fas positive immunohistochemical staining was present in the intestinal mucosa (ANP 0.5 h); **D:** Fas positive immunohistochemical staining was present in the intestinal mucosa (ANP 12 h).

at 0.5 and 2 h, suggesting that a pro-inflammatory response was predominant in these rats and significantly lower in P group at 6 h suggesting that an anti-inflammatory response was predominant. In the sFas assay, there was no significant difference between P group and C group. Interestingly, IL-2, IL-10 and sFas levels were moderate at 0.5 h, peaked at 6 h and decreased at 12 h. In our model, the intestinal tissue injury which was assessed according to the standard scale of pathological examination was closely paralleled by Fas expression and dynamic changes of IL-2/IL-10.

In conclusion, the abnormal apoptosis of Fas can significantly affect the cytokine. Fas were involved in the pathogenesis of intestinal injury in ANP. The mechanisms of Fas may have been related to Fas mediated T helper cell apoptosis.

COMMENTS

Background

Acute pancreatitis (AP) is sudden inflammation of the pancreas that may be mild or life threatening but that usually subsides. It is generally accepted that AP is often complicated by intestinal injury. The Fas system was originally characterized as a key mechanism for inducing apoptosis in immune cells. Apoptosis is a teleologically beneficial form of cell death in AP. However, little is known about how the induction of apoptosis reduces the severity of AP. Recent research has demonstrated that a Th1 to Th2 immune deviation is beneficial to mucosal immunity.

Research frontiers

Recent research has demonstrated that a Th1 to Th2 immune deviation is beneficial to mucosal immunity. The antigen-induced deletion of Th is often accompanied by an imbalance in Th1 and Th2.

Innovations and breakthroughs

In the present study, we performed immunohistochemistry staining of apoptosis-related protein Fas and investigated dynamic changes of serum IL-2, IL-10, IL-2/IL-10 and sFas in rats with ANP.

Applications

To provide the experimental basis by expression of Fas in intestinal mucosa in rats with ANP and provide experimental evidence for the immune treatment of patients with ANP.

Terminology

Th1 and Th2 response: Th1-type cytokines tend to produce the proinflammatory responses responsible for killing intracellular parasites and for perpetuating autoimmune responses. Interferon gamma is the main Th1 cytokine. Excessive proinflammatory responses can lead to uncontrolled tissue damage, so there needs to be a mechanism to counteract this. The Th2-type cytokines include interleukins 4, 5, 13 and interleukin-10, which has more of an anti-inflammatory response.

Peer review

This is a potentially interesting study to understand the dynamic changes of several cytokines and sFas in rats with ANP and provide experimental evidence for the immune treatment of ANP patients.

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Clinicopathologic characteristics of intrahepatic cholangiocarcinoma in patients with positive serum a-fetoprotein

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Abstract

AIM: To explore clinicopathologic characteristics of intrahepatic cholangiocarcinoma (ICC) in patients with positive serum a-fetoprotein (AFP).

METHODS: One hundred and thirty one patients who underwent surgical dissection for pathologically confirmed ICC were divided into a positive AFP (> 20 ng/mL) group ($n = 32$) and a negative AFP group ($n = 99$), whose clinicopathologic features were analyzed and compared.

RESULTS: The positive rate of HBsAg and liver cirrhosis of the positive AFP group was higher than that of the negative AFP group, while the positive rate of CA19-9 (> 37 U/mL) and the lymph node metastasis rate was lower.

CONCLUSION: ICC patients with positive AFP share many clinicopathologic similarities with hepatocellular carcinoma.

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Key words: Intrahepatic cholangiocarcinoma; A-fetoprotein; Hepatitis B virus; Liver cirrhosis; Hepatic stem cells

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INTRODUCTION

Intrahepatic cholangiocarcinoma (ICC) is a tumor originating from peripheral intrahepatic biliary epithelia, ranking as the second most common primary hepatic malignant tumor next to hepatocellular carcinoma (HCC), accounting for 5% of all primary hepatic malignant tumors^[1]. The incidence and mortality of ICC is on the rise in recent years^[2]. Serum a-fetoprotein (AFP), as a tumor marker of HCC^[3-5], and carbohydrate antigen 19-9 (CA19-9), as a tumor marker of ICC, have been widely used in clinical practice^[6]. In about 19% ICC patients, serum AFP is also positive (> 20 ng/mL)^[1], but there is little knowledge about the clinicopathologic features of such patients. The purpose of this study was to define clinicopathologic features of ICC patients with positive AFP by comparing them with ICC patients with negative AFP.

MATERIALS AND METHODS

Patients

Included in this study were 131 ICC patients who received surgical dissection at the Eastern Hepatobiliary Surgery Hospital of the Second Military Medical University (Shanghai, China) from March 2002 to June 2003, including 90 males and 41 females ranging in age from 23 to 73 years with a mean of 53 years. Of the 131 ICC patients, serum AFP was positive in 32 patients (24.4%), of whom AFP was > 200 ng/mL in 13 patients (9.9%), and > 1000 ng/mL in 6 patients (4.5%). Their clinical manifestations, pathological findings and surgical outcomes were compared with those of ICC patients whose serum AFP was negative. Positive serum hepatitis B surface antigen (HBsAg) and hepatitis C antibody were biomarkers of chronic viral hepatitis.

The diagnosis of ICC was confirmed by pathology. All the excised specimens were fixed in 4% neutral formaldehyde routinely, paraffin embedded, sliced into 4 μ m sections, and haematoxylin and eosin (HE) stained.

Table 1 Clinical features of ICC patients with positive AFP

	AFP		P value
	+	-	
	(n = 32)	(n = 99)	
Gender (M/F)	24/8	66/33	NS
Age (yr)	48.7 ± 11.7	54.5 ± 9.9	0.007
HBsAg + (%)	25 (78.1)	38 (38.3)	0.000
Anti-HCV + (%)	0	1 (0.01)	NS
CA19-9 (> 37 U/mL) (%)	10 (31.4)	58 (58.6)	0.007
TBIL (> 17.1 μmol/L) (%)	17 (53.1)	39 (39.4)	NS
ALT (> 40 IU/L) (%)	16 (50.0)	26 (26.3)	0.012
AST (> 40 IU/L) (%)	14 (43.8)	23 (23.2)	0.025

NS: Not significant; M: Male; F: Female; HBsAg: Hepatitis B surface antigen; HCV: Hepatitis C virus antibody; TBIL: Total bilirubin; AST: Aspartate transaminase; ALT: Alanine transaminase.

Pathological study included the size, number and location of the tumors, background of cirrhosis, portal or hepatic vein invasion, lymph node metastasis, formation of tumor capsules, and histological grade. Tumors whose diameter was smaller than 3 cm were classified as small ICC.

Immunohistochemistry for HCC marker hepatocyte paraffin 1 (Hep Par 1)^[7] and ICC marker cytokeratin 19 (CK-19)^[8] was performed using a polymer-based method with the Envision Kit (Fuzhou Maxim Biotech, China). Formalin-fixed, paraffin-embedded serial tissue sections (4 μm) were deparaffinised and rehydrated in xylene and grade-diluted ethanol. Tissue sections were then incubated in methanol containing 0.3% hydrogen peroxide at room temperature for 20 min to block endogenous peroxidase. Sections were then incubated overnight at 4°C with anti-Hep Par 1 antibody (Dako, Denmark) or anti-CK-19 antibody (NeoMarkers, USA), followed by incubation with Envision reagent at room temperature for 30 min, and color developed with 3, 3'-diaminobenzidine tetrahydrochloride. Finally, the sections were counterstained with haematoxylin, and hyalinized water. For negative controls, the sections were processed the same way, except they were incubated with phosphate-buffered saline instead of the primary antibody.

All patients were followed up after discharge from the hospital, with a median follow-up period of 31 mo (range 5-52 mo).

Statistical analysis

Data were analyzed with SPSS 11.0 statistical software. Quantitative inter-group comparison was tested by *t* test, and classification inter-group comparison was tested by χ^2 test. Survival analysis was done by Kaplan-Meier method. Inter-group comparison was done by log-rank method. *P* < 0.05 was considered statistically significant.

RESULTS

Clinical features

The mean age of the positive AFP group was lower than that of the negative AFP group (*P* = 0.007). There was no significant difference in sex distribution between the two groups. The positive rate of HBsAg (78.1%) and

Table 2 Pathologic features of ICC patients with and without positive AFP

	AFP		P value
	+	-	
	(n = 32)	(n = 99)	
Tumor location (%)			NS
Right lobe	21 (65.6)	57 (57.6)	
Left lobe	8 (25.0)	33 (33.3)	
Both lobes	3 (9.4)	9 (9.1)	
Tumor size (cm)			NS
mean ± SD	7.97 ± 4.12	6.83 ± 2.98	
≤ 3 (%)	2 (0.6)	9 (0.9)	
Tumor number (%)			NS
Single	25 (78.2)	62 (62.7)	
≥ 2	7 (21.8)	37 (37.3)	
Liver cirrhosis (%)	13 (40.6)	22 (22.2)	0.041
Capsule formation (%)	8 (25.0)	15 (15.2)	NS
Histological grades (%)			NS
Well- Moderately	21 (65.6)	68 (68.6)	
Poorly	11 (34.4)	31 (31.4)	
Lymph node metastasis (%)	5 (15.6)	35 (35.4)	0.035
Portal invasion (%)	1 (3.1)	5 (5.1)	NS
Microvascular invasion (%)	18 (56.2)	62 (62.6)	NS
Immunohistochemical examinations			NS
Hepatocyte paraffin	1	0	0
Cytokeratin 19 (%)	32 (100)	99 (100)	

NS: Not significant.

transaminase of the positive AFP group was higher than that of the negative AFP group (*P* = 0.000 and *P* = 0.036 respectively), while the positive rate of CA19-9 was lower (> 37 U/mL, *P* = 0.007; Table 1).

Pathological features

Backgrounds of liver cirrhosis were elicited in 13 ICC patients with positive AFP, which was significantly higher than that of the negative AFP group (40.6% *vs* 22.2%, *P* = 0.041), but the lymph node metastasis rate was significantly lower (15.6% *vs* 35.4%, *P* = 0.035). There were no significant differences in the location, size and number of tumors, tumor capsule defect, histological differentiation, portal venous invasion and microvascular invasion (Table 2). Immunohistochemical staining showed that Hep Par 1 expression was negative and CK-19 expression was positive in all 131 cases (Figure 1).

Outcomes

No hospital death occurred in all the 131 ICC cases. The median postoperative survival of the ICC patients with positive AFP and with negative AFP was 37 mo and 28 mo respectively. The cumulative 1-year and 3-year survival rate of the positive AFP group was 68.7% and 46.8% respectively, both higher than 64.6% and 40.4% of the negative AFP group, though the difference was not statistically significant. Possible risk factors affecting survival included tumor size > 3 cm (*P* = 0.014), lymph node metastasis (*P* < 0.0001), portal venous invasion (*P* = 0.006), and the number of tumors ≥ 2 (*P* < 0.0001).

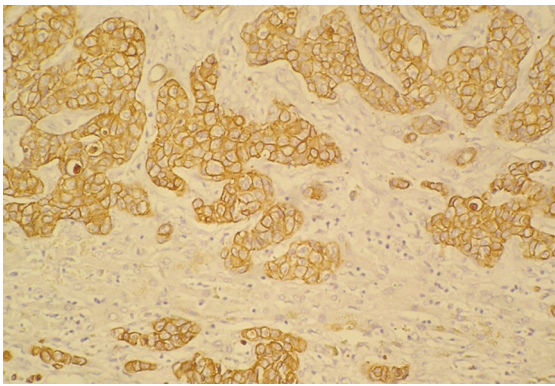


Figure 1 Representative sections showing immunohistochemical expression of CK-19 in intrahepatic cholangiocarcinoma (× 200).

DISCUSSION

Human AFP is a fetal glucoprotein with a molecular weight of about 72 kDa. Under physiological conditions, it is synthesized by fetal hepatocytes, yolk sac cells and gastrointestinal cells. AFP level begins to decrease gradually to < 10 ng/mL by 300 d of birth. Since detection of AFP in the serum of HCC patients in 1963, AFP has been widely used for screen examination and clinical diagnosis as an HCC tumor marker. In 60%-70% HCC patients, serum AFP is higher than the normal range^[1,3,4]. In addition, increased AFP is also found in other pathological conditions such as hepatic cirrhosis, extensive hepatic necrosis, chronic hepatitis, pregnancy, gonadal fetal tumors and digestive tract tumors including gastric carcinoma, pancreatic carcinoma and gallbladder carcinoma. Positive AFP is rarely seen in ICC patients. A series of studies from a Japanese liver cancer research team showed that 19% ICC patients had a serum AFP level > 20 ng/mL, 10.3% > 200 ng/mL, and only 6.3% ICC patients had a serum AFP level > 1000 ng/mL^[1]. In our series of 131 ICC patients, 32 patients (24.4%) had positive AFP, including 13 patients (9.9%) > 200 ng/mL, and 6 patients (4.5%) > 1000 ng/mL. The exact mechanism of how AFP is synthesized in ICC is not clear.

We found that ICC patients with positive AFP were associated with HBV infection and cirrhosis. This clinical feature is similar to that of HCC. What is consistent with ICC is that transaminase (a biomarker reflecting hepatic impairment) was higher in the positive AFP group than in the negative AFP group. Yamamoto *et al*^[9] reported that ICC patients who were preoperatively diagnosed as having HCC had a relatively high rate of HCV infection. In ICC patients presenting with a high level of AFP and a low level of CA19-9, surgical treatment similar to HCC should be considered. Okuda *et al*^[10] found that in ICC patients with positive Lens culinaris agglutinin-A-reactive AFP (AFP-L3), the hepatitis viruses infection rate was as high as 60%.

Lymph node metastasis is a common event in ICC; while it occurs rarely in HCC^[11]. The data obtained from our study showed that the lymph node metastasis rate was low in ICC patients with positive AFP. What is consistent with previous studies is that lymph node metastasis is an important factor affecting the prognosis

of ICC^[12]. We found that the 1-year and 3-year survival rate of the positive AFP group was higher than that of the negative AFP group. This may be due to the lower lymph node metastasis rate of ICC patients with positive AFP. However, as the capacity of our cases is small and the follow-up period is not long enough, this statistical difference may not be significant.

The pathogenesis of ICC remains unclear. Recent studies show that HCC, ICC and many other tumors may originate from stem cells^[13]. It is generally accepted that adult hepatic stem cells are hepatic oval cells. They are a group of intrahepatobiliary multi-potential differentiation cells, capable of differentiating to hepatobiliary cells and to hepatic cells. These cells are mainly located in the fetal liver or the hepatobiliary terminal Hering tube in adults. In normal physiological conditions, the number of oval cells is very small, and they are in a resting state. When the hepatic parenchyma is severely damaged, or regeneration of the hepatic cells is inhibited by virus, drugs, hepatic toxins or carcinogens, oval cells are activated, proliferating in large numbers and differentiating to hepatic and hepatobiliary cells to repair and reconstruct the liver^[13]. AFP is not only an indicator of cell de-differentiation or immaturity but an important sign of hepatic stem cells^[14]. Wang *et al*^[15] reported that the expression rate of hepatic stem cell marker CK7 and CK19 was 100% in 12 ICC patients, while the expression rate of c-kit, Thy-1 and AFP was 41.7%, 33.3% and 33.3%, respectively. Transformation of oval cells to ICC cells was also observed. AFP synthesis in ICC suggests that ICC may originate from hepatic stem cells that underwent malignant transformation^[16]. However, this presumption awaits verification by more studies.

Liver fluke infection (*Clonorchis sinensis* or *Opisthorchi viverrini*)^[17,18], primary sclerosing cholangitis (PSC)^[19,20], and hepatolithiasis are thought to be the risk factors for ICC^[21,22]. Multiple studies in recent years show that viral hepatitis and hepatic cirrhosis are not only closely related to HCC but to ICC^[23-26]. Our most recent case-control study showed that HBV infection is the possible pathogenic factor causing ICC in Chinese populations^[27]. Proliferation of large numbers of oval cells was seen in chronic HBV and hepatic cirrhosis^[28-30]. HBV infection may induce activation of oval cells, and this process may be accompanied with abnormal genetic alteration^[31], which in turn triggers malignant transformation of oval cells.

In summary, ICC patients with positive AFP share many clinicopathologic similarities with HCC.

COMMENTS

Background

Intrahepatic cholangiocarcinoma (ICC) is the second most common primary hepatic malignant tumor next to hepatocellular carcinoma (HCC). Serum a-fetoprotein (AFP), as a tumor marker of HCC, has been widely used in clinical practice. In about 19% ICC patients, serum AFP is also positive (> 20 ng/mL), but there is little knowledge about the clinicopathologic features of such patients.

Research frontiers

One hundred and thirty one patients who underwent surgical dissection for pathologically confirmed ICC were divided into a positive AFP (> 20 ng/mL) group (n = 32) and a negative AFP group (n = 99), whose clinicopathologic features were analyzed and compared.

Innovations and breakthroughs

The positive rate of HBsAg and liver cirrhosis of the positive AFP group was higher than that of the negative AFP group, while the positive rate of CA19-9 (> 37 U/mL) and the lymph node metastasis rate was lower.

Applications

AFP synthesis in ICC suggests that ICC may originate from hepatic stem cells that underwent malignant transformation^[16]. However, this presumption awaits verification by more studies.

Peer review

This paper by Yan-Ming Zhou is an interesting study that describes the clinicopathologic characteristics of patients affected by intrahepatic cholangiocarcinoma with positive and negative serum AFP. This study includes 131 patients who underwent surgical dissection for pathologically confirmed ICC. The authors, concluding that ICC patients with positive AFP share many clinicopathologic similarities with HCC, suggest new perspectives in the management of intrahepatic cholangiocarcinoma.

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Risk factors for alcohol-related liver injury in the island population of China: A population-based case-control study

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Abstract

AIM: To investigate the association of alcohol dose, duration of drinking and obesity with abnormal alcohol-related liver injury indicators, the prevalence of alcohol-related liver injury in the island population of China.

METHODS: Randomized multistage stratified cluster sampling from the island population of China was used in the population-based case-control study. Then interview, physical examination, laboratory assessments and ultrasonography were done.

RESULTS: Daily alcohol intake ≥ 20 g, duration of drinking ≥ 5 years and obesity were closely related to alcohol-related liver injury ($P < 0.05$). The odds-ratio (OR) (95% CI) was 1.965 (1.122-3.442), 3.412 (1.789-6.507) and 1.887 (1.261-2.824), respectively. The prevalence rate of alcohol-related liver injury in ≥ 20 g daily alcohol intake group and < 20 g daily alcohol intake group was 37.14% and 12.06%, respectively. The prevalence rate of alcohol-related liver injury in ≥ 5 years drinking group and < 5 years drinking group was 34.44% and 8.53%, respectively. No significant dose-response relation was found between daily alcohol intake and abnormal alcohol-related liver injury indicators as well as between duration of drinking and abnormal alcohol-related liver injury indicators. There was no significant difference in the prevalence of alcohol-related liver injury between

beer drinking group and yellow rice wine drinking group, hard liquor drinking group, multiple drinking group.

CONCLUSION: The risk threshold of daily alcohol intake is 20 g and duration of drinking inducing alcohol-related liver injury 5 years in the island population of China. Liver injury induced by obesity should be concerned.

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Key words: Alcohol; Liver injury; Prevalence; Case-control study; Epidemiology

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INTRODUCTION

Alcohol-induced liver disease remains one of the most common causes of chronic liver diseases^[1]. Studies on alcoholic liver disease (ALD) have drawn wide attention in the Western world^[2-5]. It was reported that ALD should be defined as an alcohol-associated lifestyle disease^[6]. The predisposition to ALD is largely governed by gene-environment interactions. In recent years, along with the improved living standard and increased alcohol consumption, several epidemiological surveys showed that it has become a serious public health problem in China^[7-10]. The island population in East China is a specific cluster of population. They feed themselves mainly on fishing, spend most of their time on sea-going ships, and consume a large amount of alcohol compared to the inland population. However, few population-based ALD studies are available from islands in China. Therefore, it is currently difficult to evaluate alcohol-related liver injury in the island population. Certainly, it is extremely important to select a sensitive and specific indicator in epidemiological survey. ALD is characterized by elevated serum gamma-glutamyltranspeptidase (GGT), aspartate aminotransferase

(AST) and alanine aminotransferase (ALT)^[11-13]. According to the Practical Guidelines for Alcoholic Liver Disease published by the American College of Gastroenterology in 1998^[14], GGT, AST and ALT were used as indicators of alcohol-related liver injury in this survey. We conducted a population-based case-control study to investigate the association of alcohol dose, duration of drinking and obesity with alcohol-related liver injury in East China.

MATERIALS AND METHODS

Study design and sample selection

We assigned a number to each of the counties is located along the coast of Zhejiang Province, and randomly selected one county (Xiangshan County). We randomly selected two islands (Hepu and Dongmen) of the 5 islands in the Xiangshan County and 9 villages of the 12 villages in the Hepu and Dongmen islands from August 2006 to September 2006. All individuals investigated in this study were at the age of over 18 years. All procedures were approved by the Ethics Committee of Zhejiang University School of Medicine. Each method and potential risks were explained in detail to the participants who gave their written informed consent before the survey.

Through a stratified multistage probability cluster sampling method, we acquired a representative sample from the island population in Zhejiang Province. We investigated 814 individuals aged 18 years or more in this survey, and obtained the complete data on 782 individuals. However, 129 individuals reporting clinical diagnosis of chronic viral hepatitis, schistosomiasis japonica (according to their epidemiological history, enzyme-linked immunosorbent assay results), cirrhosis (according to their medical history and ultrasonography results), or other severe diseases (mainly including drug-induced liver disease, cancer, pancreatitis, kidney disease, *etc*, based on their medical history) were excluded. Therefore, complete data were collected from 653 individuals. Their HBsAg and anti-HCV were negative. All individuals had no history of drug-induced liver disease and other severe diseases. There was no significant difference in the mean age between males and females. The mean age of males and females was 50.11 ± 13.48 years and 50.56 ± 11.72 years, respectively. There was also no significant difference in the mean BMI between males and females. The mean BMI of males and female was 24.60 ± 3.83 kg/m² and 24.54 ± 3.58 kg/m², respectively.

Interview

A face-to-face interview was conducted by trained physicians using a standardized questionnaire at the local community hospital. Data on demographic variables, alcohol drinking status, medical history and health behavior were collected from the questionnaire. Educational attainment of the followed up individuals was categorized into 5 groups according to the years of education (0, 1-6, 7-9, 10-12, ≥ 12 years). Based on smoking habit grading^[15], the followed up individuals were categorized into non-smoker group (never and cessation of smoking for more than 6 mo), smoking addict group (daily smoking for more than 6 mo),

and smoking non-addict group (cessation of smoking or daily smoking for less than 6 mo). A series of questions of alcohol use included quantity of alcohol intake each time, times of alcohol intake each day, months of alcohol intake each year, years of alcohol intake, types and alcoholicity of alcoholic beverage, drinking and dietary habits. From the above data, we calculated the average daily alcohol intake (g), total alcohol intake (kg), and duration of drinking (years) by alcohol dose convert formula^[16].

Physical examination

All followed up individuals were invited to have a physical examination at the local community hospital after the face-to-face interview. The followed up individuals were required to fast overnight. Body measurements were performed by a trained medical professional using a standardized protocol. Body weight and standing height were measured in light indoor clothing without shoes. Body mass index (BMI) was then calculated as mass (kg)/height (m)². The followed up individuals were divided into non-obese (BMI < 25 kg/m²) group or obese (BMI ≥ 25 kg/m²) group as previously described^[17]. Blood pressure was measured with an electronic blood pressure monitor (Omron HEM-746C, Omron Healthcare Inc., Bannockburn, Illinois, USA) on the right arm of the followed up individuals at a comfortable sitting position after a 5-min rest. Three measurements were taken. The second and third pressure readings were averaged and used for analysis. Diagnosis of hypertension was based on The JNC 7 Report^[18] or on the current use of anti-hypertensive medications.

Laboratory assessments

Peripheral venous blood samples were collected after physical examination and centrifuged at 3000 r/min for 15 min at 4°C. After being frozen, the samples were shipped on dry ice to Department of Clinical Laboratory, First Affiliated Hospital, School of Medicine, Zhejiang University, and stored at -80°C. Blood samples were taken to check alcohol-related liver injury indicators which reflect the changes in the alcohol-related liver injury^[14,16], including ALT, AST, GGT. Also, HBsAg, anti-HCV and enzyme-linked immunosorbent assay (ELISA) for schistosomiasis japonica were detected. All serum biochemistries were measured with a Hitachi 7600-110 automatic analyzer (Hitachi co., Tokyo, Japan). Reference value ranges of all indexes were based on the biochemistry criteria of Department of Clinical Laboratory, First Affiliated Hospital, School of Medicine, Zhejiang University. According to the Practical Guidelines for Alcoholic Liver Disease published by the American College of Gastroenterology in 1998^[14], abnormal alcohol-related liver injury indicators were defined based upon AST > ALT (ALT or AST exceeding the upper normal level) or GGT exceeding the upper normal level.

Ultrasonographic examination

Hepatic ultrasonography for all individuals was performed by the same experienced ultrasonographer using a GE Logic Book XP portable ultrasound with a 3.5 MHz probe. Ultrasonographic diagnosis of cirrhosis followed the ultrasonographic criteria^[19].

Table 1 Relationship between variables and alcohol-related liver injury detected by using univariate logistic-regression

Variable	β	S.E.	Wald χ^2	P	OR	95% CI
Male gender	1.152	0.228	25.448	0.000	3.163	2.022-4.948
Age	-0.008	0.007	1.162	0.281	0.992	0.978-1.006
Education level	0.061	0.127	0.229	0.632	1.063	0.829-1.362
Unmarried state	-0.144	0.408	0.125	0.724	0.866	0.389-1.926
Smoking	0.810	0.190	18.184	0.000	2.248	1.549-3.262
Daily alcohol intake ≥ 20 g	1.460	0.201	52.562	0.000	4.307	2.902-6.392
Duration of drinking ≥ 5 years	1.729	0.237	53.324	0.000	5.633	3.542-8.958
Total alcohol intake ≥ 36.5 kg	1.506	0.208	52.582	0.000	4.507	3.000-6.711
Hypertension	0.473	0.209	5.128	0.024	1.605	1.066-2.418
Obesity	0.671	0.188	12.673	0.000	1.956	1.352-2.829

Table 2 Multivariate logistic-regression analysis of alcohol-related liver injury and selected variables

Variable	β	S.E.	Wald χ^2	P	OR	95% CI
Daily alcohol intake ≥ 20 g	0.676	0.286	5.584	0.018	1.965	1.122-3.442
Duration of drinking ≥ 5 years	1.227	0.329	13.884	0.000	3.412	1.789-6.507
Obesity	0.635	0.206	9.518	0.002	1.887	1.261-2.824

Table 3 Prevalence rate of alcohol-related liver injury (%)

Variable	Total ALI	ALI in obese	ALI in non-obese
Daily alcohol intake(g)			
< 20	45/373 (12.06)	22/157 (14.01)	23/216 (10.65)
≥ 20	104/280 (37.14)	60/119 (50.42)	44/161 (27.33)
Duration of drinking (yr)			
<5	25/293 (8.53)	12/118 (10.17)	13/175 (7.43)
≥ 5	124/360 (34.44)	70/158 (44.30)	54/202 (26.73)

ALI: Alcohol-related liver injury.

Statistical analysis

We established a database using Epi Data 3.0 software (The EpiData Association, Odense, Denmark). Two typists recorded the data respectively and checked each other, corrected errors until two pieces of data were consistent. Statistical analysis was performed with SPSS 13.0 statistical package (SPSS Inc., Chicago, Illinois, USA). The mean value for different groups was compared using Student *t*-test. Chi-square (χ^2) test was used for comparing group ratios. We carried out univariate and multivariate stepwise logistic regression analyses. $P < 0.05$ was considered statistically significant.

RESULTS

Risk factors for abnormal alcohol-related liver injury indicators

Of the 653 individuals, 149 were diagnosed having abnormal alcohol-related liver injury indicators in this study. Univariate logistic-regression analysis showed that male gender, smoking, ≥ 20 g daily alcohol intake, ≥ 5 years drinking, ≥ 36.5 kg total alcohol intake, hypertension, obesity were closely related to abnormal alcohol-related liver injury indicators, while age, education level, unmarried state were not significantly related to abnormal alcohol-related liver injury indicators (Table 1).

Multivariate stepwise logistic-regression analysis showed that ≥ 20 g daily alcohol intake, ≥ 5 years drinking and obesity were closely related to abnormal alcohol-related liver injury indicators (Table 2). Compared to the < 20 g daily alcohol intake group, the odds-ratio (OR, 95% CI) of abnormal alcohol-related liver injury indicators in the ≥ 20 g daily alcohol intake group was 1.965 (1.122-3.442). Compared to the < 5 years drinking group, the OR (95% CI) of abnormal alcohol-related liver injury indicators in the ≥ 5 years drinking group was 3.412 (1.789-6.507).

Table 4 Relationship between different daily alcohol intake and BMI

Group	ALI	Normal	χ^2	P	OR	95%CI
X ₀	23	193	-	-	-	-
X ₁	44	117	17.564	0.000	3.156	1.813-5.492
X ₂	22	135	0.970	0.325	1.367	0.732-2.553
X ₃	60	59	65.121	0.000	8.534	4.864-14.972
Y ₀	13	162	-	-	-	-
Y ₁	54	148	23.911	0.000	4.547	2.385-8.668
Y ₂	12	106	0.678	0.410	1.411	0.620-3.209
Y ₃	70	88	60.338	0.000	9.913	5.194-18.918

X₀: Daily alcohol intake < 20 g and BMI < 25 kg/m²; X₁: Daily alcohol intake ≥ 20 g and BMI < 25 kg/m² group; X₂: Daily alcohol intake < 20 g and BMI ≥ 25 kg/m²; X₃: Daily alcohol intake ≥ 20 g and BMI ≥ 25 kg/m². Y₀: Drinking time < 5 years and BMI < 25 kg/m²; Y₁: Drinking time ≥ 5 years and BMI < 25 kg/m²; Y₂: Drinking time < 5 years and BMI ≥ 25 kg/m²; Y₃: Drinking time ≥ 5 years and BMI ≥ 25 kg/m².

Prevalence of alcohol-related liver injury

Based on the daily alcohol intake and BMI, 216 subjects were assigned to control group (daily alcohol intake < 20 g and BMI < 25 kg/m²), 161 to excessive drinking group (daily alcohol intake ≥ 20 g and BMI < 25 kg/m²), 157 to obese group (daily alcohol intake < 20 g and BMI ≥ 25 kg/m²), 119 to excessive drinking and obese group (daily alcohol intake ≥ 20 g and BMI ≥ 25 kg/m²). The prevalence rate of abnormal alcohol-related liver injury indicators in the four groups was 10.7%, 27.3%, 14.0% and 50.4%, respectively (Table 3). Compared to the control group, the OR (95% CI) of abnormal alcohol-related liver injury indicators in the other groups was 3.156 (1.813-5.492, $P = 0.000$), 1.367 (0.732-2.553, $P = 0.325$), 8.534 (4.864-14.972, $P = 0.000$), respectively (Table 4).

Table 5 Dose-response to daily alcohol intake, drinking time and alcohol-related liver injury

Characteristics	Total	ALI	χ^2	P	OR (95%CI)	PR (%)	PR
Daily alcohol intake (g)							
< 20	373	45	-	-	-	12.06	1.00
20-40	76	25	20.817	0.000	3.573 (2.019-6.324)	32.89	2.73
40-80	73	27	28.011	0.000	4.278 (2.424-7.552)	36.99	3.07
80-160	65	24	25.774	0.000	4.267 (2.360-7.715)	36.92	3.06
≥ 160	66	28	37.283	0.000	5.371 (3.010-9.584)	42.42	3.52
Duration of drinking (yr)							
< 5	293	25	-	-	-	8.53	1.00
5-10	22	4	2.279	0.131	2.382 (0.748-7.587)	18.18	2.13
10-20	58	22	36.082	0.000	6.551 (3.351-12.806)	37.93	4.45
20-40	206	76	60.265	0.000	6.267 (3.808-10.313)	36.89	4.32
≥ 40	74	22	23.773	0.000	4.535 (2.379-8.647)	29.73	3.49

ALI: Alcohol-related liver injury; PR (%): Prevalence rate; PR: Prevalence ratio.

Based on the duration of drinking and BMI, 175 subjects were assigned to control group (duration of drinking < 5 years and BMI < 25 kg/m²), 202 subjects to long-term drinking group (duration of drinking ≥ 5 years and BMI < 25 kg/m²), 118 to obese group (duration of drinking < 5 years and BMI ≥ 25 kg/m²), 158 to long-term drinking and obese group (duration of drinking ≥ 5 years and BMI ≥ 25 kg/m²). The prevalence rate of abnormal alcohol-related liver injury indicators in the four groups was 7.4%, 26.7%, 10.2% and 44.3%, respectively (Table 3). Compared to the control group, the OR (95% CI) of abnormal alcohol-related liver injury indicators in the other groups was 4.547 (2.385-8.668, $P = 0.000$), 1.411 (0.620-3.209, $P = 0.410$), 9.913 (5.194-18.918, $P = 0.000$), respectively (Table 4).

Dose-response relation of alcohol intake with alcohol-related liver injury

Compared to the < 20 g daily alcohol intake group, the OR of abnormal alcohol-related liver injury indicators in the other groups (daily alcohol intake was 20-40 g, 40-80 g, 80-160 g, ≥ 160 g, respectively) was 3.573 ($P = 0.000$), 4.278 ($P = 0.000$), 4.267 ($P = 0.000$), 5.371 ($P = 0.000$), respectively (Table 5). The prevalence rate of abnormal alcohol-related liver injury indicators in the other groups was 12.1%, 32.9%, 37.0%, 36.9%, 42.4%, respectively. Compared to the < 20 g daily alcohol intake group, the prevalence rate in the other groups was 2.73, 3.07, 3.06 and 3.52, respectively. Compared to the < 5 years drinking group, the OR of abnormal alcohol-related liver injury indicators in the other groups (drinking duration was 5-10 years, 10-20 years, 20-40 years, ≥ 40 years) was 2.382 ($P = 0.131$), 6.551 ($P = 0.000$), 6.267 ($P = 0.000$), 4.535 ($P = 0.000$), respectively (Table 5). The prevalence rate of abnormal alcohol-related liver injury indicators in the other groups was 8.5%, 18.2%, 37.9%, 36.9%, 29.7%, respectively. Compared to the < 5 years drinking group, the prevalence ratio in the other groups was 2.13, 4.45, 4.32 and 3.49, respectively.

Different types of alcoholic beverage and alcohol-related liver injury

Of the 313 drinkers, 126 were beer drinkers, 36 yellow rice

Table 6 Different types of alcoholic beverage and alcohol-related liver injury

Type of beverage	Total	ALI (n, %)	Daily intake (g)	χ^2	P
Beer	126	38 (30.16)	40.26 ± 31.35	-	-
Yellow rice wine	36	14 (38.89)	78.15 ± 57.88	0.979	0.322
Hard liquor	41	16 (39.02)	107.09 ± 84.38	1.111	0.292
Multiple	110	44 (40.00)	167.26 ± 109.60	2.509	0.113
Total	313	112 (35.78)			

ALI: Alcohol-related liver injury.

wine drinkers, 41 hard liquor drinkers and 110 multiple drinkers. The prevalence rate of abnormal alcohol-related liver injury indicators in these four kinds of drinkers was 30.2%, 38.9%, 39.0% and 40.0%, respectively. Compared to the beer drinkers, no significant difference was found in the prevalence rate of abnormal alcohol-related liver injury indicators among the other groups (Table 6).

Analysis of obesity

Obesity (BMI ≥ 25 kg/m²) was also found to be an important risk factor for liver injury (Tables 1 and 2). Multivariate stepwise logistic-regression analysis showed that the OR (95% CI) of abnormal alcohol-related liver injury indicators was 1.887 (1.261-2.824) in the BMI ≥ 25 kg/m² group compared to the BMI < 25 kg/m² group (Table 2).

DISCUSSION

Liver is the major alcohol processing organ. Chronic heavy drinking induces liver injury and results in alcoholic liver disease, even irreversible alcoholic liver cirrhosis^[20]. Since ALD has no specific clinical features^[21] and no specific laboratory tests^[14] are available for it, its diagnosis is currently based on drinking history, related laboratory assessments and imaging^[16,22,23]. Certainly, liver biopsy is the gold standard for diagnosis of ALD^[24], but it is hard to use this invasive examination in population-based epidemiological surveys. Irie *et al*^[11] found that GGT synthesis and protein expression are increased in ALD, leading to elevated serum levels of GGT

that are commonly noted in patients with the disease. Elevated GGT is somewhat more sensitive at 69%-73% with a specificity of 65%-80% for excessive alcohol consumption^[25,26]. An elevated serum AST in relation to serum ALT has been proposed as an indicator of alcohol-induced organ damage^[27]. It was reported that most patients with high alcohol consumption but without severe liver disease do not have an AST/ALT ratio above one. A high AST/ALT ratio suggests advanced alcoholic liver disease^[13]. Therefore, we chose the GGT and AST/ALT ratio as the indicators of alcohol-related liver injury in this epidemiological survey.

In the present study, logistic-regression analysis demonstrated that daily alcohol intake ≥ 20 g, drinking time ≥ 5 years and obesity were important risks for abnormal alcohol-related liver injury indicators. The risk for abnormal alcohol-related liver injury indicators was 1.965-fold higher in the ≥ 20 g daily alcohol intake group than in the < 20 g daily alcohol intake group, while the risk for abnormal alcohol-related liver injury indicators was 3.412-fold higher in the ≥ 5 years drinking group than in the < 5 years drinking group. However, the risk thresholds of alcohol intake-induced alcoholic liver injury were different in different areas. No uniformed conclusion has been achieved in alcohol intake-induced alcoholic liver injury^[28-32]. It was reported that even low alcohol level is a significant risk of developing liver disease^[33]. Therefore, significant differences exist among different racial and ethnic groups and even in different individuals^[34,35]. It has been shown that genotype of ethanol metabolizing enzyme genes in the Chinese population is different from Western population^[36]. Therefore, genetic factors in the island population from East China need to be further studied.

As to the dose-response relation of alcohol intake and abnormal alcohol-related liver injury indicators, our results demonstrate that there was no significant dose-response relation between daily alcohol intake, drinking time and abnormal alcohol-related liver injury indicators. Kamper-Jorgensen *et al.*^[37] showed that alcoholic threshold has a greater effect on the mortality of alcoholic cirrhosis.

Obesity is also an important risk factor for liver injury. In this study, logistic-regression analysis showed that the risk of obesity for abnormal alcohol-related liver injury indicators was 1.887. Some studies found that obesity is an independent risk factor for liver injury in alcohol drinkers^[38-40]. In our study, the prevalence rate of alcohol-related liver injury in the non-obese group, non-excessive drinking or no long-term drinking group was lower than that in the obese group, excessive drinking group or long-term drinking group. The highest prevalence rate of alcohol-related liver injury was found in the obese and excessive drinking/long-term drinking group. There was a difference in the odds-ratio of abnormal alcohol-related liver injury indicators between the non-obese and excessive drinking/long-term drinking group, the obese and excessive drinking/long-term drinking group and control group ($P < 0.05$), while was no difference in the odds-ratio of abnormal alcohol-related liver injury indicators between the obese and non-excessive drinking group and no long-

term drinking group, suggesting that the specificity of GGT and AST/ALT ratio can be used as the indicators of alcohol-related liver injury.

However, hepatotoxic consequences of obesity and ethanol ingestion have important prognostic implications and might be useful to formulate body mass index-based guidelines for "safe" alcohol consumption^[41].

No significant difference was found in the morbidity of abnormal alcohol-related liver injury indicators between the beer and other groups, suggesting that the types of alcoholic beverage are not closely related with abnormal alcohol-related liver injury indicators. Therefore, we believe that alcohol intake plays a more significant role in liver injury than the type of alcoholic beverage.

The island population from East China is a specific cluster of population. They feed themselves mainly on fishing and spend most of their time on sea-going ships. Although they consume a large amount of alcohol every day, their alcohol-related liver injury is not very severe. Most individuals in this area are alcohol drinkers. It was reported that treatment modalities aiming at reducing alcohol intake in alcohol-dependent patients include psychological, pharmacological and psychological therapies, but many patients benefit more from pharmacological therapy^[42]. We believe that epidemiology study of the island population from East China is more important than that of the inland population. Certainly, it is more useful to analyze the differences in island and inland populations, including drinking habit, diet habit, living and working pressure, genotype, *etc.* ALD is governed by gene, environmental and psychological factors. We will continue to pay close attention to the island population from East China.

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COMMENTS

Background

The island population from East China is a specific cluster of population. They consume a large amount of alcohol compared to the inland population. However, few population-based studies on alcoholic liver disease (ALD) are available from islands in China. We conducted a population-based case-control study to investigate the association of alcohol consumption, drinking time and obesity with liver injury in the island population from East China.

Research frontiers

The association of alcohol consumption and drinking time with alcohol-related liver injury in the island population from East China was studied.

Innovations and breakthroughs

The risk threshold of daily alcohol intake is 20 g and the drinking time that induces alcohol-related liver injury is 5 years in the island population from East China. Obesity-induced liver injury should also be concerned. Whether hepatotoxic consequences of obesity and alcohol ingestion are additive or synergistic is worthy to be further studied.

Applications

The results are useful to analyze the differences in the island and inland

population, including drinking habit, diet habit, living and working pressure, genotype.

Terminology

Gamma-glutamyltranspeptidase (GGT) and aspartate aminotransferase/ alanine aminotransferase (AST/ALT) ratio are the characteristics of alcoholic liver disease (ALD). Abnormal alcohol-related liver injury indicators are defined based upon the condition that aspartate aminotransferase / alanine aminotransferase (AST>ALT) (ALT or AST exceeding the upper normal level) or GGT exceeding the upper normal level.

Peer review

The paper describes the association of alcohol consumption and drinking time with alcohol-related liver injury in the island population from East China. The topic is highly interesting. The island population should be concerned in the follow-up research in future.

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RAPID COMMUNICATION

Endoscopic diagnosis of gastrointestinal graft-versus-host disease

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Abstract

AIM: To evaluate the diagnostic value of endoscopy in patients with gastrointestinal graft-versus-host disease (GI GVHD).

METHODS: We identified 8 patients with GI GVHD following allogeneic hematopoietic stem cell transplantation (HSCT). GVHD was defined histologically as the presence of gland apoptosis, not explained by other inflammatory or infectious etiologies.

RESULTS: The symptoms of GI GVHD included anorexia, nausea, vomiting, watery diarrhea, abdominal pain, GI bleeding, etc. Upper endoscopic appearance varied from subtle mucosal edema, hyperemia, erythema to obvious erosion. Colonoscopic examination showed diffuse edema, hyperemia, patchy erosion, scattered ulcer, sloughing and active bleeding. Histological changes in GI GVHD included apoptosis of crypt epithelial cells, dropout of crypts, and lymphocytic infiltration in epithelium and lamina propria. The involvement of stomach and rectocolon varied from diffuse to focal.

CONCLUSION: Endoscopy may play a significant role in early diagnosis of GI GVHD patients following allogeneic HSCT, and histologic examination of gastrointestinal biopsies is needed to confirm the final diagnosis.

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Key words: Gastrointestinal graft-versus-host disease; Endoscopy; Diagnosis; Allogeneic hematopoietic stem cell transplantation

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INTRODUCTION

Allogeneic hematopoietic stem cell transplantation (HSCT) is increasingly performed for a variety of disorders, including acute and chronic leukemia, hematologic malignancies, and marrow failure states^[1]. Graft-versus-host disease (GVHD) is the leading cause of morbidity and mortality after allogeneic HSCT^[2,3]. Gastrointestinal (GI) complaints are relatively common within the first 100 d following allogeneic HSCT^[4,5]. Although nausea, vomiting, anorexia and high-volume diarrhea are the common manifestations of GI GVHD, they may also be attributable to chemoradiation toxicity, medication side effects, or a variety of bacterial, fungal, viral infections. Thus, it is very difficult to establish the diagnosis of GI GVHD based on the clinical grounds alone^[6].

Endoscopy with biopsy has been shown to be accurate in the identification of GVHD. Although previous reports have documented a high yield for rectal biopsy^[7,8], upper GI biopsies are superior to rectal or rectosigmoid biopsies in the diagnosis of GVHD^[9,10]. Thus further evaluation may be needed to establish the best diagnostic approach to GI GVHD.

Our aim in the present study was to demonstrate the endoscopic and histological features of GI GVHD. Eight patients with proven GI GVHD were included in the study, and we intended to evaluate the significance of endoscopy and biopsy in the diagnosis of GI-GVHD.

MATERIALS AND METHODS

Patients

From January 2002 to December 2006, eight patients with suspected GI GVHD 20 d following allogeneic HSCT at the First Affiliated Hospital of Soochow University were enrolled in this study. All patients were interviewed and the following data were recorded: age, gender, underlying disease and transplantation type, stool per day, stool volume, nausea, vomiting, diarrhea, anorexia, gastrointestinal bleeding and skin rash. Laboratory studies including liver chemistry tests were also recorded. Routine

Table 1 Characteristics of patients with GVHD

Case No./Sex/Age	Diagnosis	Conditioning regimens	Donor HLA match	GVHD prophylaxis	Stage grading
1/F/29	AML-M2	BU/CY	HLA 2-locus mismatched unrelated donor	CSA, ATG, MMFMTX	aGVHD grade IV
2/M/47	CML-CR	Fludarabine, Bu, ATG	HLA-identical sibling donor	CSA, MTX	cGVHD Limited
3/M/39	CML-CR	BU/CY	HLA-identical sibling donor	CSA, MTX	cGVHD/Limited
4/M/23	CML-CR	BU/CY	HLA-identical sibling donor	CSA, MTX	aGVHD grade IV
5/M/63	CML-CR	Fludarabine, Bu, ATG	HLA-identical sibling donor	CSA, MTX	aGVHD grade IV
6/F/35	ALL-CR	Me-CCNU, TBI, Ara-C, CY	HLA 2-locus mismatched related donor	CSA, MTX, MMF, ATG	aGVHD grade IV
7/M/42	ALL-CR	BU/CY, Me-CCNU, Ara-C	HLA 2-locus mismatched related donor	CSA, MTX, MMF, ATG	aGVHD grade III
8/F/23	ALL-CR	Me-CCNU, TBI, Ara-C, CY	HLA 2-locus mismatched related donor	CSA, MTX, MMF, ATG Anti-CD25	aGVHD grade III

HLA: Human leucocyte antigen.

stool examination and bacterial culture were performed for all patients. Cytomegalovirus (CMV) antigenemia was monitored twice weekly after conditioning regimens.

Histocompatibility and stem cell source

One patient with acute myeloid leukemia (AML) underwent 2-locus mismatched unrelated donor transplant. Four patients with chronic myeloid leukemia (CML) were recipients of a matched related donor (MRD) transplant. Three patients with acute lymphoblastic leukemia (ALL) received haploid related donor transplant. Peripheral blood hematopoietic stem cells were collected from donors in all but one case. One case with AML had hematopoietic stem cells harvested from bone marrow through Taiwan Marrow Donor Registry.

Conditioning regimen and GVHD prophylaxis

Following conditioning regimens were used: BuCy (busulfan 4 mg/kg per day for 4 d and cyclophosphamide 60 mg/kg per day for 2 d) for standard transplantation in patients 1, 3 and 4; Bu-Fludara-ATG (antihuman thymocyte globulin) (busulfan 4 mg/kg per day for 2 d, fludarabine 30 mg/m² per day for 6 d, and antithymocyte globulin 2.5 mg/kg per day for 4 d) for non-myeloablative transplantation in patients 2, 5; Me-CCNU (semustine)-TBI (total body radiation)-Cy (Me-CCNU 250 mg/m², -d₈; TBI 8Gy, -d₁; Ara-C (arabinosylcytosin) 4 g/m², -d₆, -d₅; Cy 1.8 g/m², -d₄, -d₃) for all ALL patients.

All patients received cyclosporine A (CSA) with short-course MTX (methotrexate) for the prophylaxis of GVHD. For patient 1 who underwent 2-locus mismatched unrelated transplantation, ATG and MMF (mycophenolic mofetil) were added. Treatment for patients with ALL was intensified and prolonged by using the combination of cyclosporine A, MMF, ATG and anti-CD25 antibody for GVHD prophylaxis.

Diagnostic criteria and GVHD grading system

GI GVHD could be diagnosed according to its clinical manifestations, endoscopic appearance and histopathological evaluation. Medication-induced side effects, chemoradiation toxicity or GI infections must be excluded. Specific histological criteria could establish the diagnosis of GI GVHD. Focal dropout and apoptosis of GI crypt epithelial cells are usually regarded as golden standard to diagnose GVHD. Acute GVHD

(aGVHD) is defined as occurring within 20 to 100 d after transplantation and chronic GVHD (cGVHD) occurring 100 d after transplantation^[5]. A clinical grading system based upon the degree of involvement for each of the organ systems was originally developed by investigators in Seattle^[2]: (1) grade I: 500-1000 cc stool/d, accompanied with anorexia and vomiting; (2) grade II: 1000-1500 cc stool/d, histologically proven GVHD by endoscopic biopsies; (3) grade III: 1500-2000 cc stool/d; (4) grade IV: over 2000 cc stool/d, accompanied with ileus and severe abdominal pain.

Gastrointestinal endoscopy and biopsy

If patients had persistent unexplained GI symptoms (diarrhea, nausea, vomiting, anorexia, abdominal pain or gastrointestinal bleeding) after transplantation, then upper endoscopy and/or colonoscopy were performed. Upper endoscopy with gastric biopsies of both antrum and body were performed in one patient, colonoscopy was performed with multiple biopsies of the ileum, right colon and rectosigmoid colon in 6 patients. A combination of upper endoscopy with colonoscopy and multiple biopsies was performed in another patient. For each patient, biopsies were systematically performed in the GI tract, two of which were transmitted to the microbiology department and studied further for bacterial, viral, or fungal pathogens. Another two biopsy specimens were immediately snap-frozen in liquid nitrogen and used for CMV immunohistochemical study. The remaining two biopsy specimens were fixed in formaldehyde, and further processed for paraffin embedding. Paraffin blocks were sectioned at 4 µm and stained with hematoxylin and eosin for routine histopathological examination.

RESULTS

Clinical presentation of GVHD

Of the eight patients, two developed grade III acute GI GVHD, and four grade IV acute GI GVHD, the remaining suffered from limited chronic GI GVHD. Detailed data are listed in Table 1. The clinical manifestations of upper GI GVHD included nausea, vomiting, anorexia, and abdominal pain. Lower GI symptoms manifested as voluminous secretory diarrhea accompanied with abdominal bloating or pain. Three patients had intestinal bleeding, and only one patient had gastric bleeding (Table 2).

Table 2 Gastrointestinal symptoms of patients with GI GVHD

Case No. /Sex/Age	Nausea	Vomiting	Anorexia	Abdominal pain	Diarrhea	Gastrointestinal bleeding
1/F/29	+	+	+	+	+	+
2/M/47	+	+	+	-	-	-
3/M/39	-	-	-	-	+	+
4/M/23	+	+	-	+	+	+
5/M/63	-	-	-	+	+	-
6/F/35	+	-	-	+	+	+
7/M/42	+	-	+	+	+	-
8/F/23	+	+	-	-	+	-

Endoscopic findings

The endoscopic findings varied greatly. The first endoscopy for patient 1 with grade IV acute GVHD showed diffuse erythema with mucosal oozing in the antrum and body of the stomach (Figure 1A). Because nausea, vomiting, melena and hematemesis persisted despite empiric treatment, emergency upper endoscopy and biopsy were repeated 1 wk later. The endoscopic appearance revealed a pale mucosal surface with reticulated submucosal small vessels accompanied with erosion and erythema in the antrum (Figure 1B). For the same patient, colonoscopy was performed after gastric bleeding was controlled, and disclosed extensive mucosal hyperemia and edema in the colon. In patient 2 with chronic GI GVHD, the upper endoscopic examination showed subtle mucosal edema with erythema in the antrum, but the appearance of the esophagus and duodenum was grossly normal. In patient 3 with chronic GVHD five months after transplantation, colonoscopic examination disclosed hemorrhagic spots, patchy erosions, and active bleeding. Patients 4, 5 and 6 showed similar diffuse damages, namely widespread erythema, multiple erosions and small ulcer. Two of the three patients had active bleeding in the colon (Figure 2A). Hemorrhagic spots and multiple shallow ulcers could be detected on the surface of rectocolon (Figure 2B). Patients 7 and 8 demonstrated widespread edema, erythema with multiple erosions without active bleeding in the total rectocolon.

Pathologic findings

In patient 1, histologic examination of gastric biopsy specimens showed focal dropout of crypt epithelial cells, variable lymphocytic infiltration of the epithelium and lamina propria, and colonic biopsies showed nonspecific inflammation. Gastric biopsies disclosed a crypt with multiple apoptotic cells in patient 2. Extensive mucosal erosions, shallow ulcer, sloughing and apoptosis of epithelial cells were found in patient 3. Extensive colonic mucosal erosion and necrosis were observed in patients 4 and 5, and biopsies of the colon in these patients showed clear histological evidence of acute GVHD. Biopsy specimens from patients 6, 7 and 8 illustrated numerous apoptotic bodies in crypts, and small lymphocytic infiltration of the adjacent lamina propria. CMV infection was not confirmed on biopsy specimens from seven patients by immunohistochemical study except for one patient with HLA 2-locus mismatched, in which colonic mucosa was weakly positive, but late antigen was negative

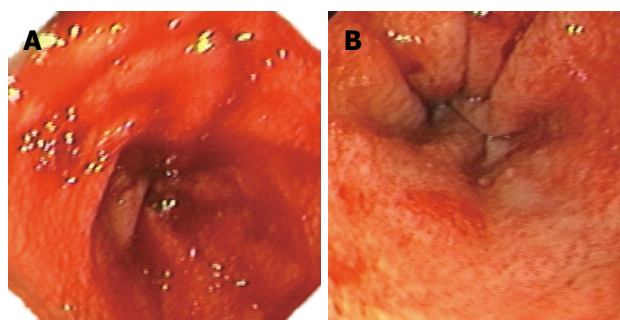


Figure 1 Upper endoscopy showing diffuse and active bleeding in the antrum and body of stomach 160 d after allo-BMT (A) and reticulated submucosal small vessels accompanied with erosion and erythema in the antrum 175 d after allo-BMT (B) in patient 1 with AML.

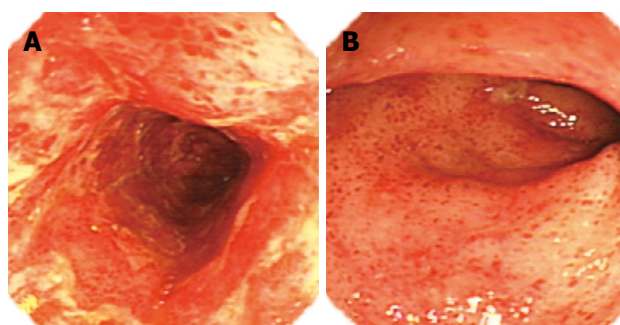


Figure 2 Colonoscopy disclosing mucosal erythema, severe erosions, multiple oozing and sloughing in ascending colon (A) and extensive hemorrhagic spots, patchy erosions, and focal shallow ulcers in the rectum (B) in patient 4 with CML 90 d after allo-HSCT.

205 d after transplantation. Because this patient had concomitantly severe GI GVHD and skin involvement 24 d after allogeneic bone marrow transplantation (BMT), GI GVHD coexisted with CMV infection.

DISCUSSION

GVHD is the leading cause of morbidity and mortality after allogeneic HSCT, occurring in up to 75% of patients^[11]. According to the degree of involvement in each of the organ systems, acute GVHD can be clinically classified as grades I-IV. High risk factors include HLA disparity, unrelated-donor transplantation, donor-recipient gender difference, old age, and infection^[12]. In the present study, one young female patient who underwent two-locus HLA-mismatched unrelated BMT suffered from grade IV acute GI GVHD 24 d after transplantation.

The principal organs with involvement of acute GI GVHD include stomach, small intestine, and rectocolon^[13], but esophageal acute GVHD is uncommon^[14,15]. Roy *et al*^[10] found that GVHD limited to the upper GI tract accounts for 18% of patients, GVHD involving the lower and upper GI tract accounts for 10%, and 26% of patients. The most prominent symptoms of GVHD involving the upper GI tract are anorexia, dyspepsia, nausea, vomiting, and, occasionally, abdominal pain^[16]. Lower GI GVHD manifests as voluminous watery diarrhea (typically secretory in nature) accompanied with abdominal bloating,

ileus, and occasionally overt intestinal bleeding^[17,18]. In contrast to acute GI GVHD, chronic GI GVHD differs markedly in distribution and histopathology. Esophageal involvement of chronic GI GVHD is not uncommon, but the stomach and intestine are rarely involved^[19].

In the present study, colonoscopy disclosed scattered hemorrhagic spots and mucosal erosion in one patient with chronic GVHD.

Obviously, clinical manifestations of GI GVHD are nonspecific. There is a wide overlap of symptoms with many GI diseases. Toxicity from the regimen of cytoreductive therapy given before transplantation can cause symptoms of anorexia, nausea, vomiting, all of which are also characteristic of GVHD^[2,20]. For most conditioning regimens, this variable is less important 20 d after transplantation, when toxicity to intestinal mucosa has largely resolved. A variety of bacterial, fungal and viral infections may affect the diagnosis of GI GVHD. Clinical manifestations of intestinal bacterial infection are mainly bloody stool and pathogenic bacteria can be confirmed from excreta. Endoscopy can also disclose mucosal erosion and pus moss. Fungal infections of the GI tract have become unusual since the routine use of prophylactic fluconazole, and fungus can be identified by examining stool specimens. In addition, since clinical symptoms of enteric CMV infection can resemble GVHD, all patients must undergo viral surveillance. Histologic identification of CMV infection is less sensitive than viral culture. Therefore, viral immunohistology and culture should be done if the patient is at a high risk for CMV infection. For more sensitive detection of CMV reactivation, polymerase chain reaction is also recommended^[21,22].

As stated previously, patients with and without GI GVHD cannot be distinguished based entirely upon clinical findings. Accurate and timely diagnosis is essential, as early recognition and intervention may significantly improve the outcome^[23,24]. Endoscopy combined with tissue biopsy is usually required to establish the diagnosis of acute GI GVHD. In a retrospective study, Terdiman and colleague^[25] confirmed that acute upper GI GVHD is sensitive to many drugs if early diagnosis could be properly made. While treatment fails, upper GI GVHD may progress to lower GI. Therefore, upper GI GVHD is an early event. Our study revealed that upper endoscopic appearance of GVHD ranged from normal mucosa to erythema, erosion, ulceration and active bleeding. Normal endoscopic examinations have been reported in up to 21% of patients with histologically confirmed acute GVHD^[20]. Sloughing of the mucosa is uncommon but high specific^[26]. It is noted that discordance may be seen in different regions of the gut. In the present study, mucosal lesions in the antrum and body were more severe than those in the funds and duodenum, whereas the esophagus was less involved.

Enteric acute GVHD exhibits diffuse hyperemia, edema, erosion, and slough of mucosa, which can resemble severe ulcerative colitis^[27]. In the present study, the grossly visible mucosal damage was uneven in distribution, sometimes appearing severely abnormal in one area while being unremarkable at other locations.

Since endoscopic appearance of GVHD is also

nonspecific, endoscopic diagnosis cannot replace histopathological examinations. At present, endoscopy with biopsies remains the gold standard for the diagnosis of acute GI GVHD^[2]. Histological criteria for GVHD are the presence of epithelial single-cell apoptosis and crypt cell dropout^[28]. However, the reported mucosal site with the highest diagnostic yield (upper and/or lower) varies in studies. In a prospective study of HSCT patients with diarrhea and upper GI symptoms, Cox and his companies^[20] discovered that the positive rate of gastric mucosal biopsies was 85% in 29 GVHD patients who were confirmed by histopathology and 58% in biopsies from duodenum and rectum-sigmoid colon. In another prospective study of 24 patients undergoing both upper and lower endoscopy^[23], biopsies were obtained from the stomach, duodenum, ileum, right and rectosigmoid colon, while the biopsy site with the highest yield was the distal colon (82%), and a combination of upper endoscopy with sigmoidoscopy and colonoscopy with ileal biopsies was equivalent (94%), suggesting that multiple biopsies should be obtained from stomach, duodenum, and rectum-sigmoid colon, in order to improve the accuracy and sensitivity of diagnosis. Many factors (chemoradiation toxicity, medication side effects, particularly CMV infection), can interfere with the histologic interpretation. Proton pump inhibitor (PPI) therapy is associated with increased apoptosis in antral biopsies. Biopsy from gastric fundus rather than from antrum may be preferable for the diagnosis of upper GI GVHD^[3]. It is, therefore, important to rule out these factors in making a histologic diagnosis of GVHD after transplantation.

There is a discrepancy between endoscopic and histologic assessments of the severity of the disease^[29]. Mucosal edema and erythema that are endoscopically impressive will be subtle when corresponding biopsies are assessed microscopically. In contrast, normal mucosa may display focal crypt epithelial apoptosis characteristic of GVHD. Thus, the correlation between endoscopic and histologic findings requires further investigation.

A clinical grading system based on the degree of lower GI symptoms (diarrhea volume, *etc*) does not consider the upper GI symptoms and endoscopic findings. Thus, an alternative, revised grading system needs to be proposed that takes into account the upper GI symptoms and endoscopic findings.

Roy *et al*^[7] showed upper GI involvement is more common than lower GI in patients with GVHD confirmed by skin biopsy. Weisdorf *et al*^[30] also confirmed that 59.7% of patients with GI GVHD have skin GVHD. Therefore, endoscopy with tissue biopsies may acquire positive results in patients with negative skin biopsies. It is noted that GI GVHD is not correlated with hepatic venous occlusion diseases (VOD).

It was reported that endoscopic examination is usually safe for patients with GVHD or occasional intestinal perforation, and oozing at the biopsy site due to thrombocytopenia^[27]. Thus, a platelet count of more than $50 \times 10^9/L$ is needed before endoscopic examination.

Because of the lack of sufficient samples, diagnostic endoscopic findings need further evaluation. In addition, endoscopists should cooperate with specialists in bone

marrow transplantation to standardize the biopsy location and the number of specimens, method and time to undertake gastroscopy and/or colonoscopy^[23].

In summary, endoscopic findings are highly variable in diagnosis of GI GVHD. There is a discrepancy between endoscopic and histologic assessments of the severity of GI GVHD. Gastrointestinal biopsies are needed to confirm the diagnosis of GI GVHD.

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COMMENTS

Background

Allogeneic hematopoietic stem cell transplantation (HSCT) is increasingly performed for a variety of disorders, such as acute and chronic leukemia, but many patients undergoing HSCT develop acute graft-versus-host disease (GVHD). GVHD involving the gastrointestinal (GI) tract is common, but it is difficult to establish the diagnosis of GI GVHD because of the nonspecific GI symptoms. Recognition of GI GVHD is critical for directing its specific therapy.

Research frontiers

The diagnosis of GI GVHD often depends on an endoscopic evaluation. The endoscopic appearance of GI GVHD can range from normal to mild edema or erythema to dramatic mucosal slough, but the mucosal damage caused by chemoradiation toxicity, side effects of medications, and enteric infections with viruses, bacteria, and fungi may occur. Although endoscopy with biopsy is commonly used in the evaluation of suspected GI GVHD, the best diagnostic approach remains undefined.

Innovations and breakthroughs

There is no standardized protocol for upper or lower endoscopy, biopsy number and location. This study demonstrated that endoscopic examinations and histologic evaluation of biopsies could be used to diagnose GI GVHD. There is a discrepancy between endoscopic and histologic assessments of the severity of GI GVHD.

Applications

The present study further demonstrated the endoscopic role in diagnosing GI GVHD in patients following allogeneic HSCT, and histologic examination of GI biopsies is needed to confirm the final diagnosis.

Terminology

GVHD: a condition that occurs following bone marrow transplantation or peripheral blood stem cell transplantation, in which lymphocytes from the graft attack specific tissues in the host. The skin, gut, and liver are the most severely affected. Drugs that suppress the immune reaction, such as steroids and cyclosporin A, reduce the severity of rejection.

Peer review

The present study reported eight patients with proven GI GVHD and demonstrated the role of endoscopic examinations and histologic evaluation of biopsies in diagnosing GI GVHD, which is very important in clinical practice.

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CASE REPORT

Extraintestinal heterotopic gastric tissue simulating acute appendicitis

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Abstract

We describe the case of a 68-year-old otherwise healthy male who presented to our emergency room with signs and symptoms of acute appendicitis. Exploratory surgery revealed a normal appendix. Further examination revealed an enlarged lymph node-like mass of tissue near the appendix, in the ileocecal mesentery. This mass was removed and was found to be inflamed heterotopic gastric tissue. Although reports of heterotopic gastric tissue in the literature are common, we believe that this case represents the first report of inflamed heterotopic gastric tissue simulating appendicitis.

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Key words: Heterotopic gastric mucosa; Acute appendicitis

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INTRODUCTION

Reports of the occurrence of heterotopic gastric tissue in the medical literature are common. Von Radhen *et al*^[1] and Steele *et al*^[2] have recently published case reports that included extensive reviews of the literature on this topic. Heterotopic gastric mucosa has been described in the esophagus and in the oral cavity, as a polyp in the distal ileum, as a mass in the rectum and anus, in the scrotum,

in the hepatobiliary system, including the gallbladder and common bile duct, in the mediastinum and in the spinal cord. Typically heterotopic gastric tissue is asymptomatic. In contrast, we present a report of gastric heterotopia in a patient who presented at our institution with signs and symptoms of acute appendicitis. To our knowledge, this presentation of heterotopic gastric mucosa is unique to the medical literature.

CASE REPORT

The patient was a previously healthy 68-year-old male whose past medical history was significant only for benign prostatic hypertrophy treated with Cardura. He presented to the emergency room with approximately 6 h of right lower quadrant pain and nausea. The pain had started in the peri-umbilical area and had localized to the right lower quadrant by the time of his presentation. The patient denied having had this type of pain previously. On examination, the patient was tender in the right lower quadrant with voluntary guarding and a positive obturator sign. His white blood cell count was 10000 with 89% granulocytes. His abdominal films showed a mild ileus pattern with no evidence of obstruction or free air.

The patient was taken to the operative suite and explored through a McBurney incision. A small amount of straw colored ascites was found upon entrance to the abdomen. The appendix was exceptionally long. However, it did not show any signs of inflammation. However, near the appendix, in the ileocecal mesentery, there was what appeared to be an enlarged, inflamed lymph node. This tissue was removed and sent to pathology for permanent stains. There was no evidence of a Meckel's diverticulum upon examination of the distal two feet of ileum. The right colon was otherwise normal in appearance and by palpation.

The tissue that had been thought to be an enlarged, inflamed lymph node was removed and established by pathology to be gastric heterotopic tissue with evidence of inflammation/gastritis (Figure 1).

Postoperatively, the patient's pain resolved. He was maintained on Prevacid (30 mg once daily) due to the concern that he might have additional areas of gastric heterotopic tissue. However, a technetium-99 scan did not show any areas of uptake postoperatively and he remains asymptomatic three years postoperatively.

DISCUSSION

We are presenting what we believe to be the first case of

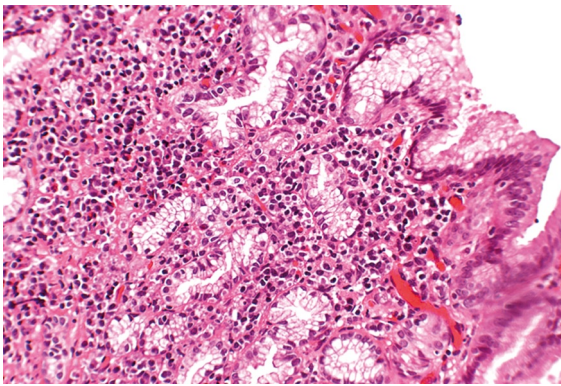


Figure 1 Gastric heterotopic tissue with evidence of inflammation/gastritis.

extraintestinal heterotopic gastric tissue with inflammation simulating appendicitis. The 2004 literature review by von Rahden *et al.*^[1] noted that heterotopic gastric mucosa has been found in the upper esophagus where it can lead to inflammation and esophageal webbing. A heterotopic gastric mucosa has also been found in Meckel's diverticula. According to Cserni^[3] the reflux-type gastritis or gastropathy in Meckel's diverticula may account for some symptoms that have prompted removal of appendixes without inflammation.

The authors have searched the literature for similar cases simulating appendicitis using search terms including (ectopic or heterotopic) and (gastric or stomach) and (abdominal pain). Case reports were found that reported patients with abdominal pain due to gastric mucosa in the gall bladder or rectum, but these were not characterized as simulating appendicitis.

Stelle *et al.*^[2] describes the two prominent theories regarding the origin of gastric heterotopic tissue. One suggests that this is congenitally displaced tissue and it therefore represents choristomas. Another leading theory suggests that the tissue originates from irregular differentiation of multipotential cells rather than displaced embryonic cells.

Regardless of the origin of the tissue, the tissue is

known to consist of gastric parietal cells capable of secreting a physiologically effective amount of acid leading to inflammation and occasionally to ulceration. Cserni^[3] notes that in most cases, the pathology does not yield evidence of *H. pylori* organisms. There was no sign of *H. pylori* in our specimen.

In conclusion we present what we believe to be the first case of extraintestinal gastric heterotopic tissue simulating appendicitis. We suggest that extraintestinal gastric heterotopia be added to the list of differential diagnoses in patients with acute abdominal pain.

COMMENTS

Background

There are no cases reported in the literature wherein heterotopic gastric tissue is described as simulating acute appendicitis.

Innovations and breakthroughs

This is new information that describes a unique presentation of heterotopic gastric tissue.

Applications

Although rare, heterotopic gastric tissue could explain pain simulating appendicitis.

Terminology

Heterotopic means located in an atypical position.

Peer review

This is a case report of extraintestinal heterotopic gastric tissue simulating appendicitis. It's very interesting and worth reporting.

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CASE REPORT

Steroid responsive eosinophilic gastric outlet obstruction in a child

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Abstract

Gastric outlet obstruction is a rare complication of eosinophilic gastroenteritis, most commonly treated surgically. We report a case of eosinophilic gastric outlet obstruction in a child that responded to conservative medical management. A brief review of this clinical entity is also provided.

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Key words: Eosinophilic gastroenteritis; Pylorus; Gastric outlet obstruction; Steroids

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INTRODUCTION

Eosinophilic gastroenteritis or gastroenteropathy (EG) is defined by variable gastrointestinal symptoms and

eosinophilic infiltration of one or more areas of the gastrointestinal tract, without evidence of parasitic or extra-intestinal disease^[1]. It can be idiopathic, related to food allergies, infections, and rarely infantile inflammatory bowel disease^[2]. A very rare complication of this entity is distal gastritis leading to gastric outlet obstruction that has been reported to occur in infancy accompanying, mimicking or generating hypertrophic pyloric stenosis^[3,4] and in adults^[5,6], but has not been described in children as an isolated manifestation of EG. Most commonly patients with eosinophilic gastric outlet obstruction have been treated surgically^[3,5,7] except for a few infantile cases where protein hydrolysate formula^[2] or steroid therapy^[4,8] has been used. Resolution of symptoms with steroid treatment has recently been demonstrated in an adult case also^[6]. We report a 2 and half-year-old Caucasian girl with eosinophilic gastric outlet obstruction treated successfully with steroids.

CASE REPORT

The previously healthy girl presented after a 3-mo history of worsening postprandial emesis leading to an inability to tolerate feedings. She had no history of atopy or diet change. She had 0.08 eosinophils ($740 \times 10^6/L$) on peripheral blood count, but was not anemic nor hypoalbuminemic. IgE level was normal. Upper gastrointestinal imaging showed marked pyloric narrowing (Figure 1). Endoscopy revealed antral edema and severe pyloric stenosis through which a Pentax 2470 endoscope (8.0 mm diameter) could not be passed. Biopsies from this area were consistent with eosinophilic gastritis (Figure 2A). Methylprednisolone (2 mg/kg per day) was started and she began tolerating liquids within two days. Endoscopy five days later revealed decreased pyloric swelling and the endoscope could be passed through the pylorus. No pyloric ulceration was seen and the duodenum appeared normal. Antral and pyloric mucosal biopsies showed resolution of the eosinophilic cellular infiltrate (Figure 2B). She was advanced to a low roughage diet within a few days, switched to oral prednisolone therapy (0.5 mg/kg per day) and discharged home. The steroids were weaned and discontinued after 8 weeks of treatment. She remained symptom free six months following the cessation of steroids.

DISCUSSION

Reported clinical manifestations of eosinophilic gastroenteritis include obstruction at various levels of the

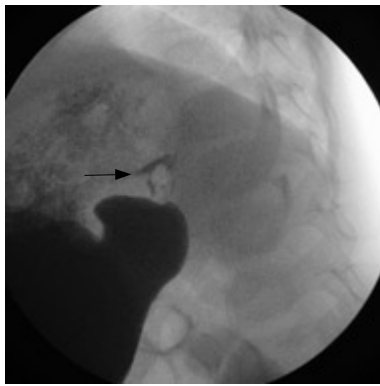


Figure 1 Upper gastrointestinal image of the patient. Note the minimal advancement of the contrast material through the pylorus (arrow).

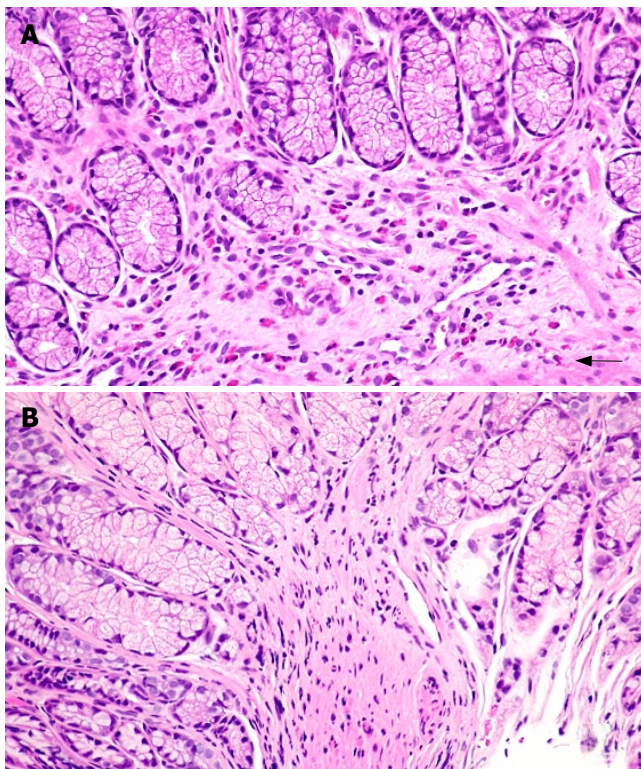


Figure 2 Histologic images before and 5 d after steroid therapy. **A:** Peripyloric antral sections showed prominent eosinophilic infiltration of the lamina propria (up to 30 eosinophils per single high power field), with occasional degranulation (arrow) of eosinophilic content and infiltration of the muscularis mucosae; **B:** Biopsies 5 d after intravenous steroid therapy demonstrated only a few eosinophils with a peak count of 2 eosinophils per high power field (HE, x 40).

gastrointestinal tract, growth failure, weight loss, anemia, melena, diarrhea, protein losing enteropathy, abdominal pain, pseudo-Crohn's disease, esophagitis, and eosinophilic ascites^[9]. On rare instances EG can even be complicated

by gastrointestinal perforation^[10]. While eosinophilic inflammation leading to pyloric stenosis and gastric outlet obstruction has been reported in adults and infants, it has not been described in children (pediatric patients more than 2 years of age) as a localized manifestation of EG to our knowledge. We could only identify one earlier case of antral web related gastric outlet obstruction that was complicated by eosinophilic inflammation in a 3-years-old child^[11]. Our patient responded briskly to steroid therapy and has remained asymptomatic for more than six months off therapy. Similar clinical response has been recorded earlier in infants with the same condition^[4,8] and very recently in an adult^[6]. However, in several instances resolution of the eosinophilic inflammation can be protracted and the course of the disease may wax and wane^[4,10]. Nevertheless, we conclude that steroid therapy should be considered in cases of eosinophilic gastric outlet obstruction prior to surgical interventions in all age groups.

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CASE REPORT

Ischemic colitis due to obstruction of mesenteric and splenic veins: A case report

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Abstract

Ischemic injury to the bowel is a well known disease entity that has a wide spectrum of pathological and clinical findings. A sudden drop in the colonic blood supply is essential to its development. We encountered a 41-year-old male patient, who presented with abdominal pain and bloody diarrhea. A colonoscopy showed markedly edematous mucosa with tortuous dilatation of the veins and a deep ulceration at the rectosigmoid junction. On an abdominal computed tomography (CT) scan and CT angiography, the mesenteric and splenic veins were absent with numerous venous collaterals for drainage. The patient gradually responded to oral aminosalicylate therapy, and was in remission after nine months. In most cases, non-occlusive ischemic injury is caused by idiopathic form and occlusive ischemia is caused by abnormalities of arteries and acute venous thrombosis. However, chronic venous insufficiency due to obstruction of macrovascular mesenteric vein rarely causes ischemia of the bowel. This report describes the first case of ischemic colitis caused by obstruction of the mesenteric and splenic veins.

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Key words: Ischemic colitis; Mesenteric vein; Splenic vein

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INTRODUCTION

Ischemic colitis is the most common ischemic injury of the gastrointestinal tract. Although it can occur at any age, approximately 90% of the patients were over 60 years of age^[1]. It is usually self-limited and is often called transient ischemic colitis.

We encountered a 41-year-old male patient, who presented with abdominal pain and bloody diarrhea, and had been diagnosed as having ulcerative colitis. The colonoscopy showed ulcerative colitis, but the rectum was spared from inflammation. Corticosteroids did not relieve the disease, but aggravated the symptoms. On an abdominal computed tomography (CT) scan and CT angiography, the mesenteric and splenic veins were absent with numerous venous collaterals for drainage. The final diagnosis was ischemic colitis with obstruction of the mesenteric and splenic veins. In this report, we describe this unusual case of ischemic colitis caused by chronic venous insufficiency.

CASE REPORT

A 41-year-old man, a Bangladeshi migrant worker in Korea, was admitted to our hospital with a three-year history of lower abdominal pain and bloody diarrhea. He was diagnosed with ulcerative colitis, treated with oral corticosteroids in an outside clinic, and referred to our facility for further evaluation. The physical examination was remarkable for lower abdominal tenderness without rebound. A routine blood analysis revealed a slightly decreased level of hemoglobin (109 g/L), hematocrit (33.8%), white cell count ($14.12 \times 10^9/L$) and platelets ($106 \times 10^9/L$). Urea and electrolytes, glucose, amylase and liver function were normal but the patient had a slightly raised level of C-reactive protein (6.7 mg/L). The patient was presumed to have ulcerative colitis with moderate to severe activity and was treated with corticosteroids. After 48 hours, he underwent a flexible sigmoidoscopy, which showed a 3-cm sized deep ulceration at the rectosigmoid junction, and friable mucosa in a diffuse circumferential distribution. Severe colitis with superimposed infection was

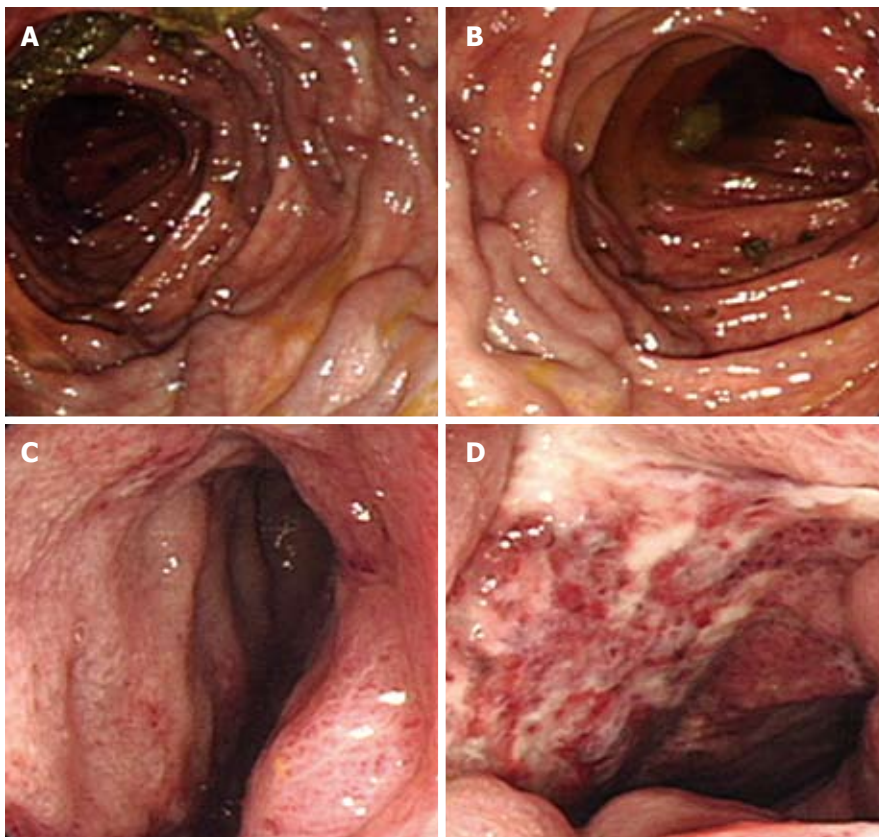


Figure 1 Colonoscopic findings. **A, B:** Tortuous and dilated submucosal veins in the colonic mucosa; **C:** Diffuse and edematous mucosa with fine granularity resembling ulcerative colitis; **D:** A deep ulceration at rectosigmoid junction.

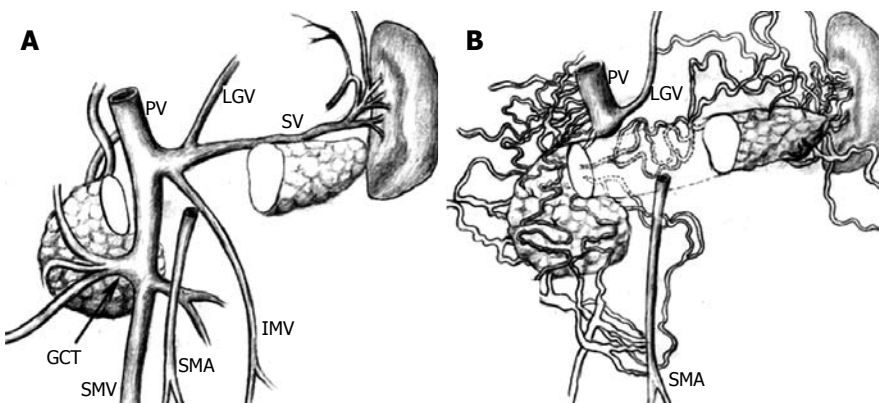


Figure 2 Dynamic abdominal CT. **A:** The normal anatomy of the main portal vein and its tributaries including the portal vein (PV), left gastric vein (LGV), gastrocolic trunk (GCT), splenic vein (SV), SMA, SMV, and IMV are schematically illustrated; **B:** The normal mesenteric vein (SMV and IMV) and splenic vein are absent. Numerous collateral venous channels developed to drain venous blood from gastrointestinal tract to portal vein.

suspected because of mild fever, and antibiotic treatment was added. Blood and stool cultures were negative. There was a minimal improvement in the clinical course, but his symptoms were not totally alleviated. Two weeks after admission, a colonoscopy was performed to evaluate the extent and other causes of the disease. It demonstrated colitis with markedly edematous mucosa and tortuous and dilated veins throughout the colon, but the rectal mucosa was spared from inflammation. The fine granularity of the mucosa was associated with friability, and contact bleeding with mucus was observed (Figure 1A-D). The biopsy specimens obtained from the edematous colonic mucosa and the ulcer of the rectosigmoid junction showed nonspecific chronic inflammation.

A dynamic abdominal CT was performed. On arterial phase CT, diffuse edematous thickening of the colon with dilated and tortuous peripheral branches of colic arteries

was seen. The rectosigmoid colon appeared markedly thickened. Major abdominal arteries including the celiac axis, splenic artery, superior mesenteric artery (SMA) and inferior mesenteric artery (IMA) were all normal in size and shape. However, on venous phase CT, the splenic vein, superior mesenteric vein (SMV) and inferior mesenteric vein (IMV) were absent. Dilated and tortuous peripheral colic veins drained into the portal vein via tortuously dilated intraperitoneal collaterals (Figures 2A and B, and 3A-C). Venous blood of gastrointestinal tract and spleen drained into the portal vein via various routes of the collateral venous pathway that are similar to the intra-abdominal venous collaterals in patients with portal hypertension. The main collateral venous routes in our patient were pericolic and mesenteric collaterals, splenic hilar and perigastric collateral-pancreaticoduodenal collaterals-the portal vein. Some venous drainage of the lower portion of the left

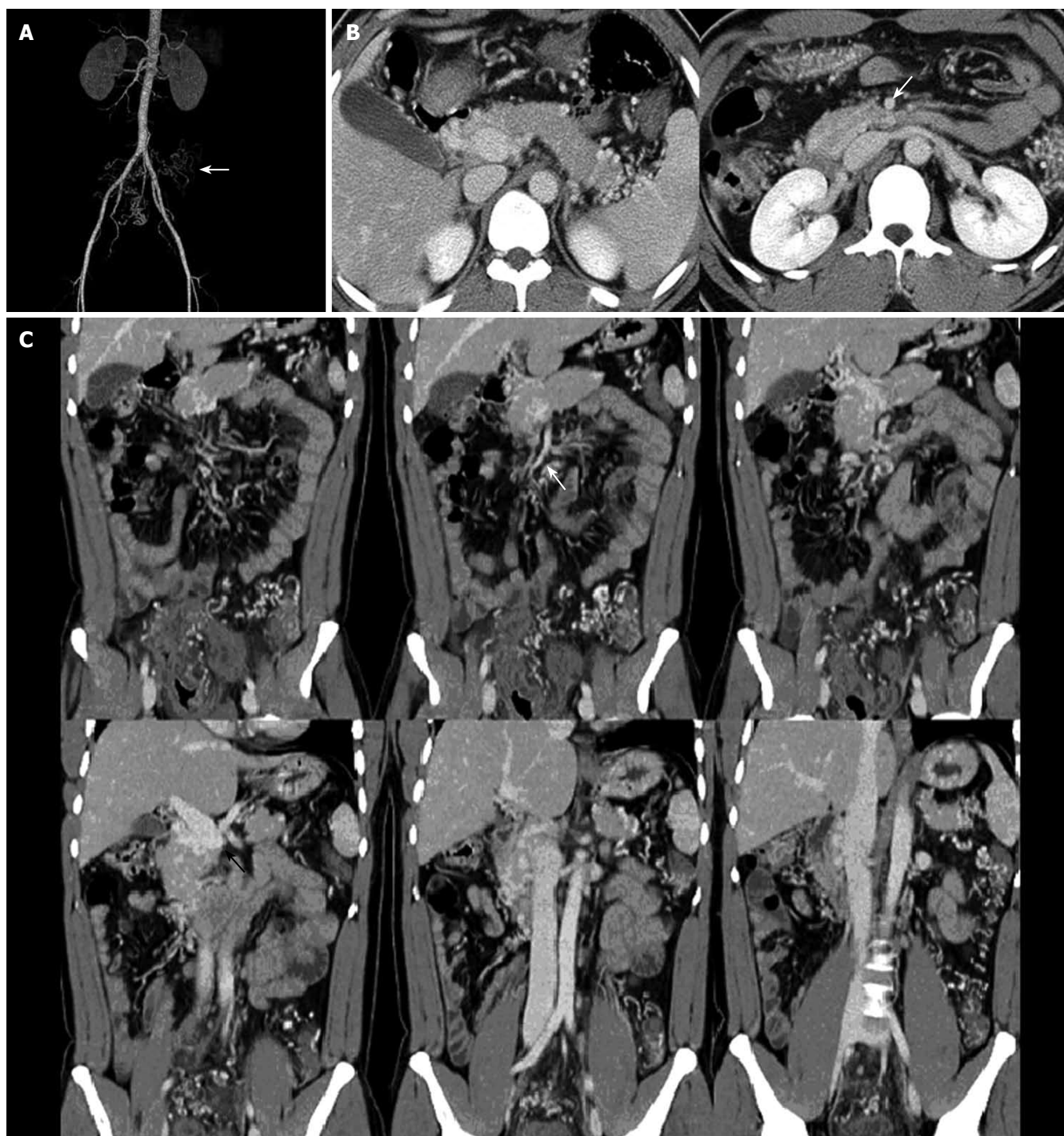


Figure 3 Abdominal CT arteriography. **A:** Normal shape and course of SMA, IMA, and splenic artery, tortuous and dilated distal branches of SMA and IMA around rectosigmoid colon; **B:** An axial and **C:** Coronal reformatted abdominal CT reveal the absent splenic vein, SMV, and IMV with numerous intra- and peripancreatic collaterals. Distal end portal vein is abruptly ended (black arrow) and SMA is normal (white arrow). Note prominent dilated pericolic arteries and venous collaterals, and markedly thickened sigmoid colon.

colon drained into the left renal vein via the left gonadal vein. In addition, the venous collaterals of the rectosigmoid colon drained into the bilateral internal iliac veins. The patient refused angiographic evaluation for the anomalous abdominal venous system.

The final diagnosis was ischemic colitis with obstruction of the mesenteric and splenic veins. Corticosteroids did not relieve the disease, but aggravated the symptoms. The patient discontinued steroid therapy and gradually

responded to oral aminosalicylates. After nine months, he was in remission and re-evaluated by sigmoidoscopy. It showed some improvement of the colonic inflammation and a complete resolution of the ulcer in the rectosigmoid area.

DISCUSSION

Ischemic colitis is a well-recognized clinical phenomenon,

although its precise etiology remains unclear. It may manifest a spectrum of severity from mild, transient mucosal erosion to fibrous scarring with stricture formation and even transmural infarction. Some cases are caused by acute macrovascular mesenteric occlusion due to surgical trauma^[2], thromboembolism^[3-5], or atherosclerosis^[6]. However, chronic venous insufficiency is rarely associated with ischemic colitis. Ischemic colitis typically develops spontaneously without signs of major vascular occlusion, and viable intestine is present elsewhere in the tract. Isolated case reports have described development of ischemic colitis in conjunction with mild allergy, hypertension, rectal prolapse, acute pancreatitis, sickle cell crisis, colon cancer, systemic lupus erythematosus, amyloidosis, anticardiolipin antibody syndrome, Buerger's disease, and Kawasaki syndrome^[7-9]. Other case reports described the association between development of ischemic colitis and use of some agents (progesterone, ergotamine derivatives, nonsteroidal anti-inflammatory drugs, and danazol)^[10], intravenous vasopressin therapy^[11], renal transplantation^[12], chronic intermittent peritoneal dialysis^[13], cocaine abuse, snake bite and marathon running^[7].

Clinical presentation is usually acute, with cramping abdominal pain of abrupt onset, abdominal distention, and bloody diarrhea. There may be local signs of peritoneal irritation over the affected segment, and if mucosal ulceration is present, bacterial invasion may also occur. However, manifestations vary widely, from severe pain with transmural infarction and early perforation to mild abdominal pain and only slight tenderness^[14].

It is extremely difficult to differentiate ischemic from ulcerative colitis. Moreover, ischemic and idiopathic ulcerative colitis may coexist^[15]. An endoscopic finding of ulcerative colitis is characterized by a uniform inflammatory reaction in the colonic mucosa, without intervening areas of normal mucosa. The majority of cases arise in the rectum, and some authorities believe that the rectum is always involved in an untreated patient^[16]. With inflammation, the mucosa becomes erythematous and granular, and the vascular pattern becomes obscured by edema. In this patient, a similar pattern of mucosal lesions was observed as mentioned above. However, while making the diagnosis as ulcerative colitis, we found that (1) the rectal mucosa was free from inflammatory reaction; (2) although the patient did not have a fulminant clinical course, a deep ulceration was observed; and (3) the disease was resistant to corticosteroid therapy and instead aggravated his clinical course. Color Doppler scans were used to differentiate the bowel-wall thickening in ischemic colitis from that seen in inflammatory bowel disease^[7].

Generally, major arterial or venous branches are easily detected on arterial or venous phase CT. The SMV is located anteriorly and to the right of the SMA and posteriorly medial to the head of the pancreas. The SMV tributaries are the ileocolonic, pancreatoduodenal, and gastroepiploic veins. The IMV originates anterior to the sacrum as the superior rectal (hemorrhoidal) vein and receives branches from the sigmoid and descending colon as it ascends to the left of midline, adjacent to the inferior mesenteric ar-

tery and left gonadal vein. In the upper abdomen, the IMV passes from posterior to the distal duodenum, anterior to the left renal vein, and then anterior to the SMA before anastomosing with the portal venous system. The splenic vein is easily detected beneath the pancreas and drains to the portal vein^[17]. In this patient, the proximal SMV, IMV and most of splenic vein were absent despite the presence of a normal SMA and IMA and splenic artery. The tortuous and dilated distal branches of SMA and IMA around the entire colon were seen on arterial phase CT and no remarkable SMV and IMV were noted on venous phase CT except for the prominent collateral veins. Instead of a normal splenic vein beneath the pancreas, tortuous splenic hilar venous collaterals developed and drained into the portal vein via the peripancreatic venous collaterals. Although we have no direct angiographic evidence, the anomaly described in this report appears unique. We presume the congenital absence of the SMV, IMV, and splenic vein results from excessive involution of proximal vitelline veins.

In this case, the arterial blood supply and venous drainage might have been balanced for a long time because of numerous abdominal venous collaterals. In addition, a possible breakage of this balance may cause venous stasis and ischemia of the gastrointestinal tract. Impaired colonic venous drainage may be a possible cause or vulnerable to the development of ischemic colitis. The relationship between the absence of mesenteric veins with possible venous stasis and ischemic colitis is not clearly established. Although the patient is currently doing well after medical and conservative treatment, a long-term follow-up is needed as there is little information in the literature regarding the outcome of the absence of the proximal mesenteric veins and its influence upon venous drainage.

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S- Editor Ma L L- Editor Ma JY E- Editor Ma WH

Successful endoscopic treatment of biliary stricture following mesenteric tear caused by blunt abdominal trauma

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Abstract

Biliary duct injuries are frequently iatrogenic, being associated with surgery for gallbladder stones. However, blunt abdominal trauma such as a motor vehicle crash is a rare cause of extrahepatic biliary stricture. A few reports have been published on biliary strictures treated with endoscopic therapy. In the present study, we describe a suprapancreatic biliary stricture associated with mesenteric tear following road traffic accident. We performed endoscopic stent placement, which was successful in relieving the biliary stricture.

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Key words: Biliary stricture; Blunt abdominal trauma; Mesenteric tear; Endoscopic stent treatment

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INTRODUCTION

Benign bile duct strictures are uncommon and usually

follow surgery for gallbladder stones, both open surgery and laparoscopic surgery^[1]. However, development of biliary stricture after blunt abdominal trauma is an extremely rare condition^[2-4]. No definitive treatment has been proposed for biliary strictures caused by blunt abdominal trauma. Recently, nonsurgical intervention using endoscopic approach has been used, with promising results^[4,5]. We present a patient with a suprapancreatic biliary stricture that developed following a mesenteric tear caused by blunt abdominal trauma due to a traffic accident. The patient was treated successfully with endoscopic plastic stent placement.

CASE REPORT

A 36-year-old man had blunt abdominal trauma due to a traffic accident. The patient was the driver and suffered full impact with the steering wheel. The patient was hospitalized for the treatment of hemoperitoneum caused by a mesenteric tear. Surgery was performed and the mesenteric tear was repaired. Two weeks after the accident, the patient developed jaundice and was transferred to our hospital for further evaluation and management.

At admission, the patient had overt jaundice with itching over the entire body. The vital signs were stable. Physical examination showed no abdominal pain, or tenderness to palpation. The bowel sounds were normal. The initial liver function tests were as follows: aspartate aminotransferase 60 U/L (normal range, 0-37 U/L), alanine aminotransferase 92 U/L (normal range, 0-41 U/L), alkaline phosphatase 317 U/L (normal range, 35-130 U/L), gamma-glutamyl transferase 107 U/L (normal range, 8-61 U/L), total bilirubin 16.9 mg/dL (normal range, 0-1.2 mg/dL), and direct bilirubin 11.3 mg/dL (normal range, 0-0.3 mg/dL). The amylase and lipase levels were within the normal range. Computer tomography (CT) scan of the abdomen revealed dilatation of both intrahepatic (IHD) and extrahepatic biliary ducts (EHD) with abrupt cut-off of the suprapancreatic portion of the common bile duct (CBD) (Figure 1). There was no evidence of pancreatitis.

An endoscopic retrograde cholangiopancreatography (ERCP) was performed to determine the cause of the jaundice. The cholangiogram revealed a 1 cm long stenosis of the distal CBD just above the pancreas, with ductal dilatation above the narrowed portion. Endoscopic biliary sphincterotomy using a pull-type papillotome and brushing and biopsy of the narrowed part were performed (Figure 2). At the end of the procedure, an endoscopic nasobiliary drain (ENBD) was placed. After the procedure, the serum

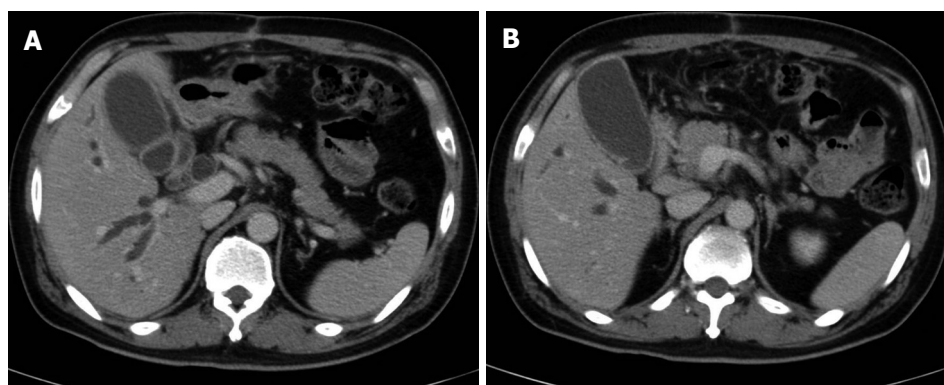


Figure 1 Abdominal CT scan findings. **A:** Dilated intra- and extrahepatic bile ducts; **B:** Abrupt cut-off of the distal common bile duct.

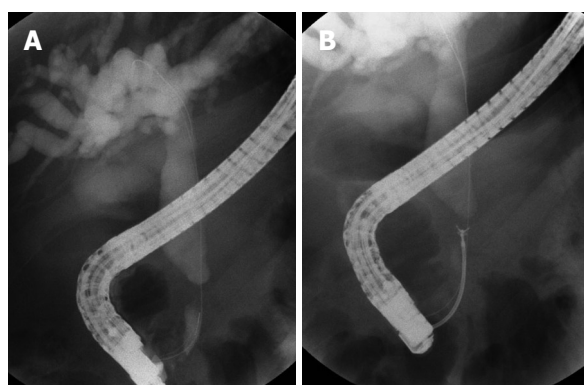


Figure 2 ERCP findings. **A:** Stenosis of the distal CBD, approximately 1 cm in length, located just above the pancreas, with ductal dilatation above the narrowed portion; **B:** Biopsy of the stricture was obtained.



Figure 4 Follow-up ERCP findings. **A:** Improvement of the CBD stricture; **B:** There is normal passage of radiocontrast material into the bile duct through the papilla.

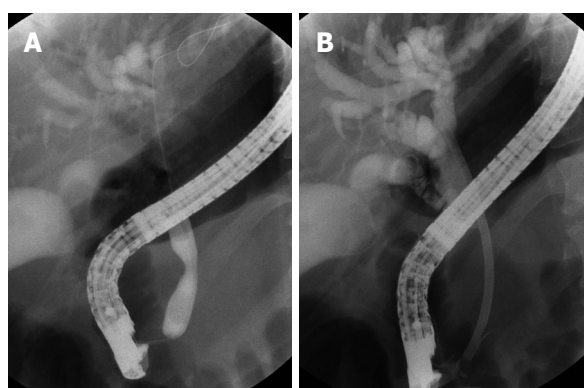


Figure 3 ERCP findings. **A:** The CBD was dilated with TTS balloons, but the dilatation was not effective; **B:** An Amsterdam-type plastic stent (12Fr, 7 cm) was placed across the stricture.

bilirubin levels decreased gradually. The cytology and biopsy results were negative for malignancy.

A repeat ERCP was performed 5 d after the first procedure and the CBD was dilated with TTS balloons. However, the stricture could not be dilated effectively. Therefore, a 12 Fr. plastic stent, 7 cm in length, was inserted and placed at the site of the lesion (Figure 3). Three months later, the patient was readmitted for a follow-up ERCP. The liver function tests were within the normal range and the patient did not have jaundice. The cholangiogram showed improvement of the CBD stricture. The plastic stent was

removed after observing normal passage of radiocontrast material (Figure 4). During a follow-up period of 26 mo, there has been no recurrence of the biliary stricture as judged by laboratory tests and radiological imaging studies.

DISCUSSION

The bile ducts are located deep within the abdomen and are protected by the ribs, liver and mesentery. Therefore, non iatrogenic bile duct injuries secondary to blunt abdominal trauma are extremely rare. It is most commonly seen following motor vehicle accidents when an unrestrained driver has an impact with the steering wheel^[2-4].

The proposed mechanisms of extrahepatic biliary strictures after blunt trauma differ based on their location. The possible mechanisms underlying the development of a suprapancreatic biliary stricture are: (1) local inflammation followed by fibrosis and stricture formation secondary to ductal tear; (2) disruption of the blood supply to the bile duct, and (3) compression of the biliary duct by an intramural or extrabiliary hematoma^[3-5]. The possible mechanisms for the development of an intrapancreatic biliary stricture include the following: posttraumatic pancreatitis with swelling of the pancreatic head, and compression of the intrapancreatic portion of the CBD; this usually resolves as the swelling of the pancreas subsides^[6]. We believe that the suprapancreatic biliary stricture in our patient was caused by disruption of the blood supply and extrinsic compression by a hematoma. The hepatobiliary area was not entered during the surgery, thus reducing the

possibility of an iatrogenic trauma.

Most patients with a biliary stricture secondary to blunt abdominal trauma develop jaundice as the initial symptom. The onset of symptoms is insidious^[3,5]. The condition may be mistaken for cholangiocarcinoma, if history of abdominal trauma is not available^[7], and may result in unnecessary surgery.

The correct diagnosis may be difficult to determine based on imaging studies such as abdominal CT and ERCP, in the absence of a complete history^[5,7,8]. Therefore, a high index of suspicion is required to ensure a proper diagnosis. To prevent irreversible fibrosis, an early diagnosis of biliary stricture is essential in order to allow timely treatment with percutaneous or endoscopic intervention^[3,5,9]. Several workers have suggested that endoscopic stent placement is an effective treatment for suprapancreatic biliary strictures caused by blunt abdominal trauma^[5,10,11]. Park *et al.*^[5] reported eight patients with suprapancreatic biliary stricture due to blunt abdominal trauma. The median length of the stricture was 1 cm. Balloon dilatation was performed in only 1 patient. The median duration of plastic stent placement was 2.9 mo. The patients were all successfully treated with the endoscopic stent placement and there was no recurrence during the follow-up period (median 33 mo). Our case was similar in terms of clinical features and outcome compared to previously reported cases. Endoscopic stenting for biliary stricture after blunt abdominal trauma is associated with low morbidity and excellent long-term outcome^[5,10,11]. Therefore, endoscopic stenting is the treatment of choice for biliary strictures that develop after blunt abdominal trauma. Surgery should only be undertaken if endoscopic intervention fails^[12,13].

In summary, we report a patient with a suprapancreatic biliary stricture secondary to blunt abdominal trauma, which was treated successfully with endoscopic stent placement. The excellent results obtained in our patient suggest that endoscopic stent placement should be considered as the primary treatment for patients who develop an extrahepatic biliary stricture after blunt abdominal trauma. Endoscopic treatment is safe and has a favorable long-term outcome.

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CASE REPORT

Appendiceal mucocoele: Case reports and review of current literature

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Abstract

The mucocoele of the appendix is an uncommon disorder which is often asymptomatic but sometimes causes acute appendicitis-like symptoms. Sometimes, patients with mucocoele can present with confusing symptoms. Preoperative suspicion and diagnosis of appendiceal mucocoele are important. Ultrasonography and computed tomography are useful tools for the diagnosis of appendiceal mucocoele. It may be also recognised by colonoscopy as a smooth submucosal lesion of the cecum. Optimal management of the mucocoele could be achieved through accurate preoperative diagnosis. Preoperative diagnosis is a major component for minimizing intra-operative and post-operative complications. We herein report five cases and discuss the diagnostic methods and surgical treatment.

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Key words: Appendix; Mucocoele; Diagnosis; Surgical treatment

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INTRODUCTION

Appendiceal mucocoele (AM) is a rare entity that can present with a variety of clinical symptoms or occur as an incidental surgical finding. The incidence is 0.2%-0.4% of all appendectomized specimens^[1-3]. AM is a progressive dilatation of the appendix from the intraluminal accumulation of the mucoid substance^[3,4]. It may be a benign or malignant process. There are four histological types, which lead to individualized surgical treatment and course in each case^[3].

Preoperative diagnosis that distinguishes AM from acute appendicitis (AA) is essential for the best choice of surgical approach (open *vs* laparoscopic) to prevent peritoneal dissemination and perform the appropriate surgery^[3,5]. Herein, we report 5 cases, discuss the diagnostic procedures and management algorithm of these patients, and review briefly the relevant literature. We also aimed to define the incidence of the AM in a tertiary referral centre in Northern Black Sea region of Turkey.

CASE REPORTS

Case 1

An 82-year-old man was admitted to the hospital because of pain in the right lower quadrant of the abdomen for 3 d. Standard laboratory tests, serum levels of CA 19-9 and carcino-embryonic antigen (CEA) were within normal ranges. Ultrasonography (USG) and computerized tomography (CT) demonstrated a well demarcated, elliptical 7 cm × 5 cm cystic mass with parietal calcifications in the right lower quadrant of the abdomen. There was an indentation in the cecum by colonoscopy. Surgical exploration revealed the mass to be an AM. Simple appendectomy was performed. Pathological examination revealed a mucinous cystadenoma with dimensions of 8 cm × 6 cm × 5.5 cm. AM restricted to the appendix and cecum was free of the disease. The patient's postoperative course was unremarkable, and he was discharged home on the 4th postoperative day.

Case 2

A 65-year-old woman was referred to the emergency de-



Figure 1 CT imaging of a soft tissue mass indicated by black arrows in the region of the cecum.

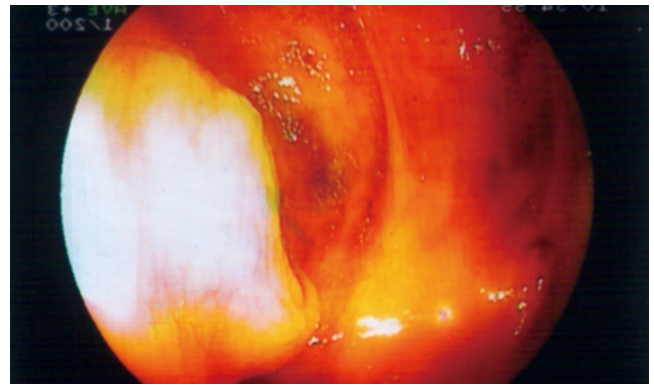


Figure 2 Colonoscopic view of the sub-mucosal mass.

partment with a diagnosis of AA. She complained of an abdominal pain which started 3 d before referral to our hospital. She had a history of coronary by-pass surgery 3 years ago. Physical examination showed diffuse peritoneal irritation. Plain radiography showed gas-fluid levels in the right lower quadrant. She also had mild leukocytosis. USG and CT scans of the abdomen showed presence of free fluid in the intra-abdominal spaces.

An emergency operation revealed ileal and cecal mesenteric ischemia. Partial ileum and cecum resection was performed. Histopathological diagnosis was ischemia of the intestine and simple mucocele of the appendix with a diameter of 5 mm, incidentally. Postoperative recovery was uneventful and she was discharged home on the 7th postoperative day.

Case 3

A 66-year-old woman was referred to the endoscopy unit for evaluation of a mass in the right lower quadrant of the abdomen with the suspicion of malignant tumor. She had visited another health care unit where the mass was revealed by ultrasound examination. She had been suffering from decreased appetite, nausea, and weakness lasting for a week. She had a history of upper GI hemorrhage managed medically 16 years ago and her surgical history was significant for cholecystectomy 14 years ago. Routine laboratory tests, including complete blood count and serum chemistry were unremarkable. Serum levels of CA19-9 and CEA were also within the normal ranges. USG of the abdomen showed a heterogenic mass (95 mm × 40 mm × 32 mm in dimensions) involving ileocecal part of the intestines. CT imaging revealed a soft tissue mass measuring 8 cm × 4 cm with peripheral enhancement in the region of the cecum (Figure 1). The patient underwent colonoscopy, which revealed a sub-mucosal protrusion to the lumen of the cecum, in the region of the appendiceal orifice. Orifice of the appendix was uncertain (Figure 2). Remaining of the colon was unremarkable. The patient was treated surgically. Surgical exploration revealed a mass in the appendix whitish-grey in color (Figure 3). Resection of cecum was followed by an ileocolic anastomosis. Frozen section examination revealed benign mucoid lesion. Formal pathologic report was hyperplastic type mucocele with evidence of secondary changes and chronic inflammatory mucosa of the intestine. The mucocele was 9.5 cm × 4 cm × 2 cm in dimensions with a wall thickness of 2

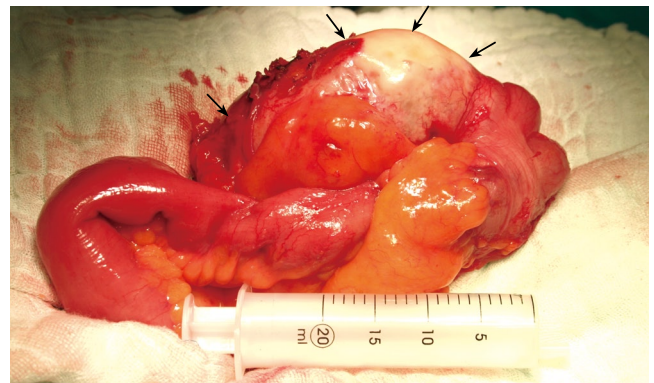


Figure 3 Intra-operative view of the AM. Arrows indicate the mucine filled appendix.

mm. There was no evidence of malignancy. Postoperative course was unremarkable and she was discharged home on the 5th postoperative day.

Case 4

A 58-year-old man was referred to the general surgery department for surgical treatment of toxic multi-nodular goitre. He had multiple previous admittances to internal medicine department and emergency department because of pain in the right lower quadrant of the abdomen and anemia. Physical examination was negative for abdominal mass or perianal or rectal lesions. The patient underwent endoscopy to investigate the etiology of anemia. Colonoscopy revealed a submucosal cecal mass. Abdominal CT revealed a polypoid a soft tissue mass measuring 4 cm × 3 cm with peripheral enhancement in the region of the cecum. The patient underwent total thyroidectomy and appendectomy. Histopathological diagnosis was benign cystadenoma without cecal involvement. Postoperative course was unremarkable and he was discharged home on the 6th postoperative day.

Case 5

A 72-year-old man underwent an open inguinal herniorrhaphy due to recurrent right inguinal hernia. He had complained of bulging and right groin pain which were exacerbated with activity. An appendiceal mass was defined during laparoscopic herniorrhaphy. Open access to the

abdominal cavity was chosen. Simple appendectomy was performed with a clinical suspicion of appendiceal mucocoele. Frozen section examination revealed appendiceal mucocoele. Pathological examination reported a mucinous cystadenoma measuring 12.5 cm and 5.5 cm without cecal involvement. Postoperative course was unremarkable and he was discharged home on the 7th postoperative day in good conditions.

DISCUSSION

Mucocoele of the appendix is an uncommon tumor, with an incidence of 0.29%-0.4% of all appendectomized specimens^[1-3,6]. There has been no exact reported incidence of AM in Turkey. Histopathological examinations revealed 5 patients with AM among 240 patients who underwent appendectomies from January 2001 to October 2007 at our institution. The incidence of AM is revealed as 2.01%, which is higher than that reported in literature^[7,8]. This may be related to the fact that our centre is a tertiary referral centre.

Although a small portion of AM is asymptomatic, clinical manifestations include right lower abdominal pain, palpable abdominal mass, or gastrointestinal bleeding in majority of the AM^[1,3,7,9-12]. Among the five cases reported above, three had abdominal pain secondary to mucocoele while one had symptoms related to groin hernia and the last patient had abdominal pain due to intestinal infarction. AM was an incidental finding for those two patients.

USG, CT and colonoscopic examinations can facilitate preoperative diagnosis of AM^[1,13-15]. Ultrasound is the first-line diagnostic modality for patients with acute abdominal pain or mass. Different sonographic findings of AM and AA have been described^[3,16,17]. Appendix diameter 15 mm or more in USG examination has been determined as the threshold for AM diagnosis with a sensitivity of 83% and a specificity of 92%^[3]. Outer diameter threshold for AA diagnosis has been established as 6 mm^[18]. USG examination revealed a cystic mass in the right lower quadrant in two of our patients. These findings revealed suspicion of AM.

CT is also an effective diagnostic tool for AM. CT can determine the relation between lesion and the neighbouring organs, and help confirm the diagnosis^[15,16,19,20]. CT reveals a cystic mass with enhancing wall nodularly in the expected area of the appendix, especially in older patients, in whom AM should be considered^[9,15,16,21]. AM could be visualized by CT in three of our patients. CT examination was normal in respect of AM for the third patient. AM was an incidental finding for this lady. Radiological investigation was not performed for the patient who had herniorrhaphy for recurrent groin hernia.

USG and CT findings are non-specific and the differential diagnosis should be established with benign appendiceal neoplasms and other pathologies such as carcinoid, lymphoma, mesenteric cysts, ovarian masses, and malign neoplasms of the appendix^[7,22]. We did not perform fine needle aspiration as dictated in the literature in order to avoid dissemination of the mucus leading to pseudomyxoma peritonei^[16].

Colonoscopy in patients with abdominal pain is a use-

ful tool for determination of mucocoele^[2,23]. Generally, an elevation of the orifice of the appendix is seen. A yellowish mucous discharge would be visible from appendiceal orifice during colonoscopy. It is also important for the diagnosis of synchronous or metachronous colon tumor which would be as high as 29%^[1,2,7,13-15,24,25]. Colonoscopic examinations on three of our patients revealed indentation in the cecum due to AM. The remaining colon was unremarkable in all of them. The 4th patient in emergency conditions was treated surgically based on the diagnosis of mesenteric ischemia and the 5th patient underwent surgery with the diagnosis of groin hernia without colonoscopic examination.

The spontaneous and surgery induced complications of AM include intestinal obstruction, intussusceptions^[20,22], intestinal bleeding^[11,13,25], fistula formation^[15,26], and volvulus^[27,28]. The worst complication is pseudomyxoma peritonei, characterized by peritoneal dissemination caused by iatrogenic or spontaneous rupture of the mucocoele^[5]. The tissues should be handled carefully during surgery in order to avoid rupture of the mucocoele. Thus, conventional surgery is preferred rather than laparoscopic approaches for the treatment in our cases^[2,5,8,22]. Laparoscopic approach has an increased risk of rupture and subsequent pseudomyxoma peritonei formation^[2,5,8]. Moreno *et al*^[5] suggest conversion to an open appendectomy in case of mucocoele when laparoscopic appendectomy is intended. Few authors still recommend a minimally invasive approach in selected patients for this rare entity^[9,27,29]. However, in these reports, laparoscopic approach has been adopted for a small number of patients. Thus, we need a large series to substantiate recommendations of laparoscopic approach.

A simple and thorough evaluation of these patients with a new algorithm has been suggested by Dhage-Ivatury and Sugarbaker^[30]. Simple appendectomy is the choice of surgical treatment for patients with benign mucocoele that has negative margins of resection without perforation. No long term follow-up is needed for these patients^[2,8,27,30]. Appendectomy was performed for three of our patients with a mucocoele limited to the appendix. For patients with perforated mucocoele, with positive margins of resection, positive cytology and positive appendiceal lymph nodes, right colectomy/cytoreductive surgery (CRS)/heated intraperitoneal chemotherapy (HIIC) and early postoperative intraperitoneal chemotherapy (EPIC) should be performed. Long term follow-up is obligatory for these patients^[5,24,30,31].

Perforated mucocoele with positive margins of resection, positive cytology, and negative appendiceal lymph nodes necessitate cecectomy/CRS/HIIC and EPIC. Long term follow-up is also obligatory for these patients^[2,30,32]. Cecectomy had been performed for one of the patients to obtain negative surgical margins, since the appendiceal wall was contiguous with the cecum and the intraoperative pathology indicated benign mucocoele. Long term follow-up in this case has been carried out in the outpatient clinics. Perforated mucocoele with positive cytology but negative margins of resection and negative appendiceal lymph nodes should be treated with appendectomy/CRS/HIIC and EPIC^[26,30]. We did not apply these treatment modal-

ties as none of our patients had had positive lymph nodes or perforated mucocele.

CONCLUSION

Although a rare disease, surgical treatment of the AM is mandatory because of the potential for malignant transformation and prevention of pseudomyxoma peritonei due to rupture of the mucocele itself. Therefore, preoperative diagnosis or suspicion is required for carefully planned resection to excise the tumor. The incidence of AM in a tertiary referral centre in Northern Black Sea region of Turkey is revealed as 2.01%, which is higher than the incidence reported in the literature.

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CASE REPORT

Tuberculous abscess in hepatoduodenal ligament: Evaluation with contrast-enhanced computed tomography

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Abstract

Two patients with tuberculous abscess in the hepatoduodenal ligament were studied. Both patients underwent contrast-enhanced computed tomography (CT) scan. The abscess showed a low density with an irregular thick wall in the hepatoduodenal ligament on CT images, the margin was poorly defined. Contrast-enhanced CT images showed the contrast-enhanced thick wall, homogeneous and peripheral-enhanced lymph nodes. Although features of the tuberculous abscess in the hepatoduodenal ligament could be conspicuously shown with contrast-enhanced CT, further experience is needed to evaluate the potential value of CT in detecting early tuberculous abscess in relation to other entities in the hepatoduodenal ligament.

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Key words: Tuberculosis; Abscess; Hepatoduodenal ligament; X-ray; Computed tomography; Lymph node

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Dong P, Wang B, Sun YQ. Tuberculous abscess in hepatoduodenal ligament: Evaluation with contrast-enhanced computer tomography. *World J Gastroenterol* 2008; 14(14): 2284-2287 Available from: URL: <http://www.wjgnet.com/1007-9327/14/2284.asp> DOI: <http://dx.doi.org/10.3748/wjg.14.2284>

INTRODUCTION

The incidence of tuberculosis is increasing^[1-4]. Abdominal

tuberculosis can affect the gastrointestinal tract, peritoneum and lymph nodes. Lymphadenopathy is the most common manifestation of abdominal tuberculosis^[5,6]. To our knowledge, there are no reports on the computed tomography (CT) appearance of tuberculous abscess in the hepatoduodenal ligament in the English radiologic literature. We describe in the paper the CT findings in a series of tuberculous abscesses in the hepatoduodenal ligament.

CASE REPORTS

Case 1

A 24-year-old man had weight loss, easy fatigability, night sweats, and obscure abdominal pains for three months, but no clinically palpable abdominal mass.

Contrast-enhanced CT was performed on a 16-detector spiral CT scanner (Siemens Sensation). The image protocol consisted of dual-phase CT scan. Medrad-100 power injector was used. Contrast material was Ultravist (Schering Germany, 300 mgI/mL). The range of CT scan covered the upper and middle abdomen. CT parameters were 120 KV, 165 eff mAs, 0.5 s/360°, 16 mm × 0.75 mm, 1 mm multi-planar reconstruction (MPR) slice width. CT scan was performed with intravenous contrast material administered as a bolus. Oral contrast material (1.2% angiografin) was administered. Images were viewed at a window width setting of 400 HU and a window level setting of 50 HU.

Contrast-enhanced CT showed a less dense mass with thick enhanced irregular walls in the hepatoduodenal ligament. The mass measured 3.9 cm × 2.8 cm with its margin poorly defined. The interface between the mass and around organs was not clear. Gas collection was not shown. Enlarged lymph nodes were detected in the portacaval space, gastrohepatic ligament, peripancreatic and upper paraaortic region. Some of them had peripheral enhancement and necrosis in the center (Figure 1A-E).

Operation on abdominal region was performed. Macroscopic pathological examination showed that the mass was tightly adherent to the hepatoduodenal ligament. Enlarged lymph nodes were found. Thirty mL of pus was aspirated from the mass. Microscopic examination revealed that the mass was inflammatory, and bacterial culture showed tuberculosis.

Case 2

A 30-year-old man had fever and obscure abdominal pain for two months with no clinically palpable abdominal mass.

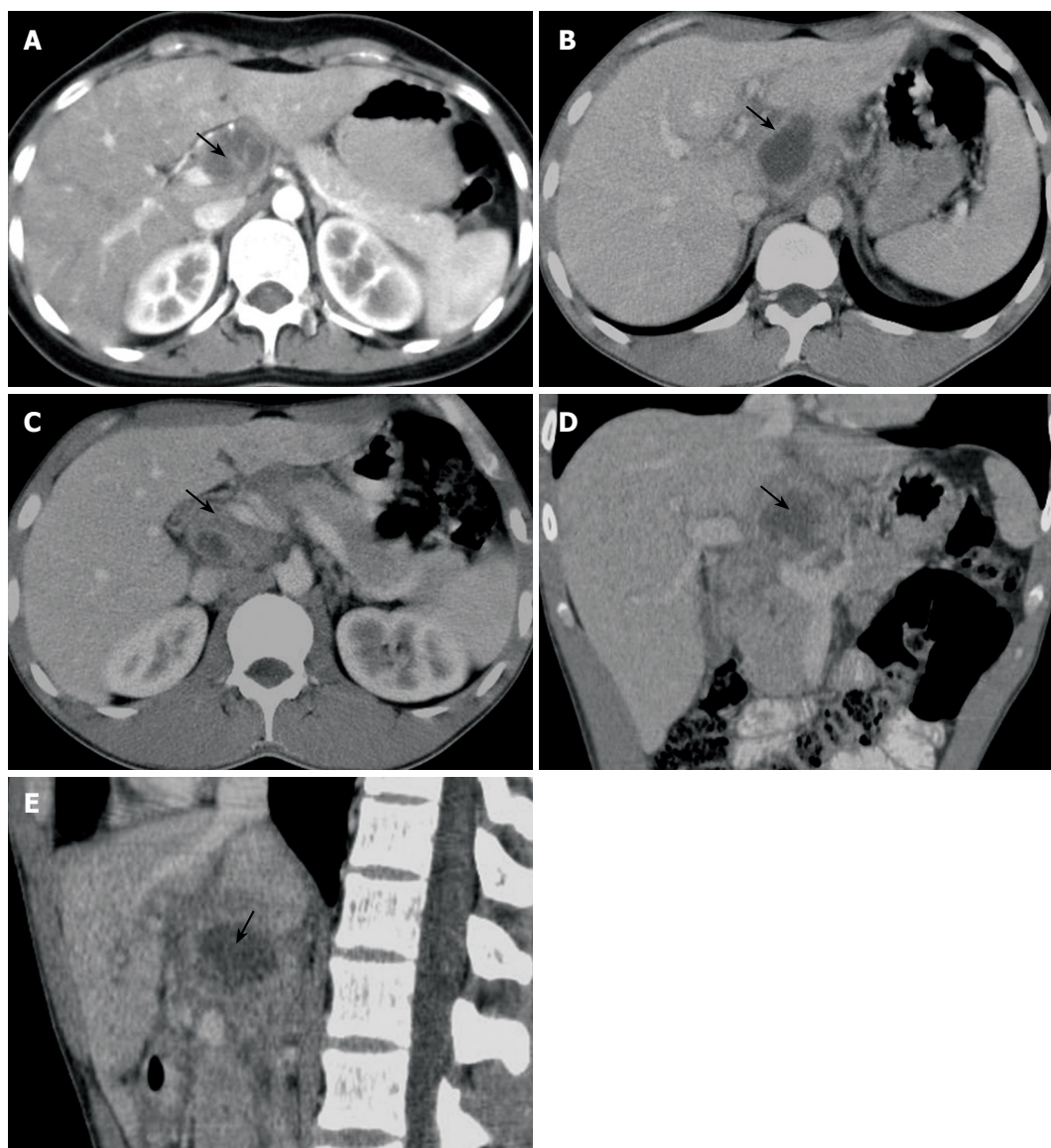


Figure 1 A 24-year-old man with tuberculous abscess in the hepatoduodenal ligament. **A:** Axial CT image (arterial phase) showing a low dense abscess measuring approximately 3.9 cm × 2.8 cm with a slightly contrast-enhanced wall in hepatoduodenal ligament (arrow); **B:** Axial CT image (venous phase) showing a low dense abscess with a contrast-enhanced wall in hepatoduodenal ligament (arrow); **C:** Axial CT image showing enlarged lymph nodes with peripheral and homogeneous enhancement in the portacaval space (arrow); **D:** Coronal CT image showing a low dense abscess with a contrast-enhanced wall in hepatoduodenal ligament (arrow); **E:** Sagittal CT image showing a low dense abscess with a contrast-enhanced wall in hepatoduodenal ligament (arrow).

Contrast-enhanced CT was performed on a spiral CT scanner (Elscent HeliCAT Flash). The image protocol consisted of venous phase. Power injector (MCT Plus, Meddred, Pittsburg) was used. Contrast material was Ultravist (Schering Germany, 300 mgI/mL). The range of CT scan covered the upper and middle abdomen. CT parameters were 120 KV, 250 mA, pitch 1. CT scan was performed with intravenous contrast material administered as a bolus. Oral contrast material (1.2% angiografin) was administered. Images were viewed at a window width setting of 400 HU and a window level setting of 50 HU.

Contrast-enhanced CT showed a less dense mass with thick enhanced irregular walls in the hepatoduodenal ligament. The mass measured 4.1 cm × 2.7 cm with its margin poorly defined. The interface between the mass and surrounding organs was not clear. Gas collection was not shown. Enlarged lymph nodes were detected in the portacaval space, gastrohepatic ligament, which had peripheral enhancement (Figure 2A and B).

Operation on abdominal region was performed. Macroscopic pathological examination showed that the mass was tightly adhered to the hepatoduodenal ligament.

Enlarged lymph nodes were found. Twenty mL of pus was aspirated from the mass. Microscopic examination revealed that the mass was tuberculosis.

Both patients gave their written informed consent.

DISCUSSION

Tuberculosis demonstrates a variety of clinical and radiologic features depending on the involved organ and has a known propensity for dissemination from its primary site. Thus, tuberculosis can mimic a number of other disease entities, and it is important to be familiar with the various radiologic features of tuberculosis to establish its diagnosis early and accurately^[7].

Lymphadenopathy is the most common manifestation of abdominal tuberculosis^[5,6]. Mesenteric, omental and peripancreatic lymph nodes are most commonly involved^[5]. Abdominal tuberculosis may be transmitted by three major routes. The first is ingestion of material infected with tubercle bacilli which are carried from a lesion in the intestinal submucosal layer to the lymph nodes draining that segment of the bowel. Drainage is usually from lymphatics



Figure 2 A 30-year-old man with a tuberculous abscess in the hepatoduodenal ligament. **A:** Axial plain CT image showing a low dense abscess measuring approximately 4.1 cm × 2.7 cm in hepatoduodenal ligament (arrow); **B:** Axial contrast-enhanced CT image showing a low dense abscess with a contrast-enhanced wall and enlarged lymph nodes in hepatoduodenal ligament (arrow).

of the ileocecum, jejunum, ileum, and right side of colon to the peripancreatic and superior mesenteric lymph nodes. The second route of transmission is hematogenous spread. Bacteria are disseminated from a distant site of infection, usually the lungs, to the abdominal lymphatic system. Because this process is systemic, it may cause infection of mesenteric lymph nodes. In the third route of transmission, infection can spread directly to the abdominal lymph nodes from the serosa of adjacent infected structures^[5]. In this study, two patients had non-disseminated tuberculosis and CT showed enlarged lymph nodes in the portacaval space, gastrohepatic ligament, peripancreatic and upper paraaortic region. So, the anatomic distribution of this disease closely parallels to the route of tuberculous infection.

The nodes are usually large and multiple, and most commonly demonstrate peripheral enhancement with central areas of low attenuation on contrast-enhanced CT images^[5]. Pathologic findings from surgically obtained specimens of tuberculous lymphadenopathy indicate that caseation or liquefactive substances at the center of enlarged lymph nodes have a low attenuation that presumably results from insufficient blood supply, whereas peripheral inflammatory lymphatic tissue has a higher attenuation on enhanced CT that results from the preserved blood supply^[8]. In this study, both cases were accompanied with enlarged lymph nodes, suggesting that the tuberculous abscess may be due to the coalescence of the involved lymph nodes.

Cystic lesions, including pseudocysts, necrotic tumors, and cysts of the pancreas and/or adjacent organs, must be differentiated from tuberculous abscess in the hepatoduodenal ligament. Pseudocyst is a unilocular, round mass with a uniform wall, and can be found in patients with clinical and laboratory evidence of pancreatitis. A well-defined rind suggests a pseudocyst or abscess, and gas bubbles suggest an abscess. CT demonstrates small calcification and fat in the teratomas located in the hepatoduodenal ligament area, and its border is round and sharp^[9-11]. CT of extension of gastrointestinal stromal tumors displays extraluminal growth, inhomogeneous enhancement, absence of calcifications and lymph node metastases^[12]. Congenital cysts (duplication, mesenteric, omental, or choledochal) may be localized to the hepatoduodenal ligament area. However, the history, clinical findings and the absence of enlarged lymph nodes do not suggest tuberculosis^[11]. Serous and mucinous cystadenomas are encapsulated and lobulated masses,

showing marked contrast-enhancement of the solid portion. Cystadenocarcinoma is seen as a multilobular, septate, thick-walled cyst or cystic neoplasm with multiple low-density areas. Dilatation of the main pancreatic duct may be seen^[10]. The radiologic pattern of tuberculous lymphadenitis can also be seen with lymphoma^[7]. The enhancement patterns of untreated lymphomas are homogeneous. In patients with lymphoma who have undergone therapy, central low attenuation may be found within nodes, simulating tuberculous lymphadenopathy. So, it is important to know the history^[13-16].

It was reported that tuberculous lymphadenopathy has the following clinical characteristics^[17]: (1) some patients have a history of TB and most of them come from areas with a high prevalence of active tuberculosis; (2) patients often suffer from epigastric pain, fever and weight loss; (3) ultrasound and CT scan show enlarged nodules, sometimes with focal calcification. In this study, a patient had weight loss, fatigability, night sweats, and obscure abdominal pains, while the other patient had fever and obscure abdominal pains. Both patients had no clinically palpable abdominal mass.

In summary, tuberculous abscess in the hepatoduodenal ligament is a less dense mass with thick enhanced irregular walls, its margin is poorly-defined, and the interface between the mass and around organs is not clear. It is important to show the peripheral enhanced lymph nodes for its early and accurate diagnosis.

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S- Editor Li DL L- Editor Wang XL E- Editor Ma WH



LETTERS TO THE EDITOR

Occult hepatitis C virus infection is more common than hepatitis B infection in maintenance hemodialysis patients

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Abstract

Patients of end stage renal disease on maintenance hemodialysis were enrolled to study the prevalence of occult and dual hepatitis B virus (HBV) and hepatitis C virus (HCV) infection and non-occult hepatitis B and C virus infection. One hundred and two patients were enrolled. Thirty patients had HCV infection, three of them were positive in anti-HCV. So, 27 (90%) of HCV-positive patients had occult HCV infection. Eleven (11%) patients had HBV infection. Five patients were positive in anti-HBc or HBV-DNA, but negative in HBsAg (occult HBV infection). Three (3%) patients had dual HBV and HCV infection. None of the patients showed changes in viral markers during the follow-up of 8 mo on average (1-12 mo).

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Key words: Occult hepatitis C; Hepatitis B; Maintenance hemodialysis

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TO THE EDITOR

We read with interest the article "Hepatitis B viral infection in maintenance hemodialysis patients: A three-year

follow-up" by Cao *et al* in 13(45): 6037-6040, 2007, *World Journal of Gastroenterology*^[1]. We agree that the hepatitis B vaccination and regular surveillance for hepatitis B virus (HBV) infection has reduced the spread of HBV in the dialysis population. The prevalence of hepatitis C virus (HCV) infection in hemodialysis (HD) patients is high and ranges from 2% to 60% between countries and among dialysis units^[2]. The prevalence of HBV and HCV occult and dual infection^[3,4] in hemodialysis patients has been variably reported.

We prospectively studied consecutive patients of end stage renal disease (ESRD) on maintenance of HD from June 2006 to June 2007 for prevalence of occult and dual hepatitis B and C virus infection and non-occult hepatitis B and C virus infection. Occult hepatitis C infection was defined as anti-HCV negative and HCV-RNA positive by polymerase chain reaction^[3,5]. All patients underwent tests of hemoglobin, urea, creatinine, bilirubin, alanine aminotransferase (ALT) and aspartate aminotransferase (AST). The viral markers done were hepatitis B surface antigen (HBsAg), antibody to hepatitis B surface antigen (anti-HBs), antibody to hepatitis B core antigen (anti-HBc), hepatitis B envelope antigen (HBeAg), antibody to hepatitis Be antigen (anti-HBe), antibody to hepatitis C virus (anti-HCV) by enzyme linked immunoassay (ELISA) and qualitatively hepatitis B virus DNA (HBV DNA) and hepatitis C virus RNA (HCV RNA) by polymerase chain reaction.

The demographic, clinical features, biochemical parameters, etiology, history of blood transfusion and time on hemodialysis are described in Table 1. One hundred and two patients were enrolled. The mean age was 41.4 years (range 17-70 years) with a male: female ratio of 68:34. The clinical presentations were generalized swelling 36 (36%), decreased urine output 34 (34%), breathlessness 30 (30%), hypertension 24 (24%) and altered sensorium in 8 patients. The mean hemoglobin, urea, creatinine, bilirubin, AST and ALT were 76.5 mg/L (33-122 mg/L) 184.3 mg/L (84-322 mg/L), 10.8 mg/L (4-23 mg/L), 0.6 mg/L (0.4-0.8 mg/L), 53.5 unit/L (26-188 unit/L) and 38.6 unit/L (16-209 unit/L). Thirty-four patients had histories of blood transfusion.

Among HD patients with HCV infection, serum ALT was elevated in 10 HCV-RNA positive patients, but normal in all the anti-HCV positive patients. Thirty (30%) patients had HCV infection, three of them had anti-HCV positivity. So, twenty-seven (90%) of HCV-positive patients had occult HCV infection.

Eleven (11%) patients had HBV infection. Five patients

Table 1 Demographics, clinical and biochemical parameters of patients on maintenance hemodialysis

Male:Female	68:34
Age (yr) ¹	41.4 (17-70)
Clinical features, cases (%)	
Generalised swelling	36 (36)
Oliguria	34 (34)
Breathlessness	30 (30)
Hypertension	24 (24)
Altered sensorium	8 (8)
Laboratory parameters ¹	
Hemoglobin (mg/L)	76.5 (33-122)
Urea (mg/L)	184.3 (84-322)
Creatinine (mg/L)	10.8 (4-23)
Bilirubin (mg/L)	0.6 (0.4-0.8)
Aspartate aminotransferase (unit/L)	53.5 (26-188)
Alanine aminotransferase (unit/L)	38.6 (16-209)
History of blood transfusion, cases (%)	34 (34)
Past history of jaundice, cases (%)	4 (4)
Etiology, cases (%)	
Chronic glomerulonephritis	44 (44)
Chronic interstitial nephritis	20 (20)
Diabetes mellitus	20 (20)
Polycystic kidney disease	5 (5)
Glomerulopathy, unknown	13 (13)
Time on hemodialysis (mo) ¹	34 (12-60)

¹Mean (range).

were positive in anti-HBc or HBV-DNA but negative in HBs Ag (occult HBV infection). Rai *et al* reported 12.2% occult HBV infection and 10.3% occult HCV infection in human immunodeficiency virus patients^[5]. Goral *et al* reported that occult HBV infection was not high in chronic HCV infected patients on HD^[6].

Three (3%) patients had dual HBV and HCV infection. Reddy *et al* found dual infection in 3.7% of patients on HD^[4]. None of the viral markers were positive in 20 patients. Four patients had past histories of jaundice, three of them had HBV infection and one was positive in HCV-RNA.

Thirty patients with positive viral markers had histories of blood transfusion ranging from 1-6 units. Agarwal *et al*^[7] showed in their studies in 208 ESRD patients with past histories of jaundice and the number of blood transfusion was significantly higher in HCV positive patients than in HCV negative patients. In our study, blood transfusion history was present in most of the patients ($n = 26$) with HCV infection. Two patients had past histories of jaundice.

On follow-up of mean 8 mo (1-12 mo), none of the patients showed change in viral markers. Twelve patients died of cardiac arrhythmias due to hyperkalemia, fluid overload due to inadequate dialysis and sepsis. In our study, the development of cirrhosis, hepatocellular carcinoma

and decompensation of liver function were not observed in HCV and HBV infected patients.

Yakaryilmaz *et al* in their group of 188 ESRD patients on maintenance of HD showed 28.7% had both occult and non-occult forms of HCV infection which was more common than HBV (19.7%) infection^[3].

HBV infection was present in 11% of patients on maintenance HD possibly due to a higher percentage (44%) of patients having protective anti-HBs titres. In the previous studies, HBV DNA positive hemodialysis patients had a significantly lower prevalence of past HBV vaccination and lower anti-HBs titres in serum than HBV DNA-negative patients of the same group^[8]. Nijhawan *et al* did the screening of 69330 subjects for HBsAg and found that prevalence of HBsAg in replacement donors was 3.1% and 2.1% in healthy voluntary donors^[9]. So, HBV infection is relatively higher in patients on HD.

So, HCV-RNA is recommended in patients on HD and now has been included in our screening program prior to renal transplantation. HBV vaccination of HD patients is an effective way of limiting the risk of transmission of HBV infection to patients on hemodialysis.

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Both manuscripts focus on the expression of Syndecan-1 and E-cadherin in differentiated type of gastric cancer, and most parts of the article published by *WJG* are exact copies of the manuscript published by *Journal of*

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Meetings

Events Calendar 2008-2009

FALK SYMPOSIA 2008

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

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

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

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

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

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

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

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

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

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

April 23-27, Milan, Italy
 43rd Annual Meeting of the European

Association for the Study of the Liver
www.easl.ch

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

May 18-21, San Diego, California, USA
 Digestive Disease Week 2008
 June 4-7, Helsinki, Finland

The 39th Nordic Meeting of Gastroenterology
www.congex.com/ngc2008
 June 6-8, Prague, Czech Republic
 3rd Annual European Meeting: Perspectives in Inflammatory Bowel Diseases
 Email: meetings@imedex.com

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

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

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

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Abstract

Opisthorchiasis caused by *Opisthorchis viverrini* (*O. viverrini*) remains a major public health problem in many parts of Southeast Asia including Thailand, Lao PDR, Vietnam and Cambodia. The infection is associated with a number of hepatobiliary diseases, including cholangitis, obstructive jaundice, hepatomegaly, cholecystitis, cholelithiasis and cholangiocarcinoma. The liver fluke infection was induced by eating raw or uncooked fish products that is the tradition and popular in the northeastern and northern region, particularly in rural areas of Thailand. Health education programs to prevent and control opisthorchiasis are still required in high-risk areas.

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Key words: *Opisthorchis viverrini*; Opisthorchiasis; Status; Thailand

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INTRODUCTION

Liver flukes are platyhelminth parasites of the class

trematoda, and *Opisthorchis viverrini* is a member of the family *Opisthorchiidae*. *Opisthorchis spp.* is a prevalent human parasite particularly in the Far East and South East Asia. *O. viverrini* is highly prevalent in Thailand and Laos while *C. sinensis* is endemic in south China, Japan, Korea and Taiwan, *O. felinus* is the prominent fluke in Eastern Europe^[1]. In Thailand *O. viverrini* is the only parasite of opisthorchiasis, the first case of opisthorchiasis was reported in 1911 by Leiper from the autopsy of a corpse in Chiang Mai. Later on Sadun in 1953, Harinasuta and Vajjarasthira in 1961, and Wykoff in 1965 had demonstrated a complete life cycle of *O. viverrini*^[2-4]. *O. viverrini* has a complicated life cycle with 2 intermediate hosts, a freshwater snail (*Bithynia goniompharus*, *B. funiculata* and *B. siamensis*) is the first intermediate host^[4,5], and a freshwater fish (*Cyclocheilichthys spp.*, *Puntius spp.*, *Hampala dispa*) is the second intermediate host where the metacercariae habitat in the muscles or under the scales. Cats, dogs, and various fish-eating mammals including humans are the definitive host^[6]. More than 7 million were infected by *O. viverrini* that were recorded by various investigators. Humans have become infected by ingesting undercooked fish containing infective metacercariae, this figure shows the tradition of eating raw or uncooked fish products as the main reason that liver flukes are a problem in Thailand. This is very popular in the northeastern and northern region particularly in rural areas. This infection is associated with a number of benign hepatobiliary diseases, including cholangitis, obstructive jaundice, hepatomegaly, cholecystitis and biliary lithiasis^[7]. Both experimental and epidemiological evidence implicate liver fluke infestation in the etiology of bile duct cancer, i.e. cholangiocarcinoma^[1,8]. In Thailand, opisthorchiasis is still a serious problem, especially in the northeast and north region. Therefore, this article investigates the distribution of the disease of the people with an emphasis on the north, north-east, central and south regions of Thailand.

HISTORY OF OPISTHORCHIASIS IN THAILAND

Fish-borne trematode in Thailand, *O. viverrini* was first described in the post-mortem examination of two prisoners from a jail in Chiangmai, northern Thailand, in 1911 by Leiper who obtained specimens from Kerr. Kerr reported that 17% of 230 adult male prisoners examined in a prison in Chiangmai were infected with *O. felinus*^[9]. In 1927, Prommas identified the worms found at an autopsy of a 17-year-old Thai male residing in Roi-et, northeast Thailand, as *O. felinus*^[10]. The liver fluke infection in

Thailand was caused by *O. viverrini*, not by *O. felinus*^[2], and Wykoff *et al* confirmed this later in 1965^[4]. Since then, cases of opisthorchiasis were reported each year. *O. viverrini* is still prevalent and a serious health problem in some parts of Thailand, therefore, the health education promotion is still required.

SOURCE OF THAI HUMAN INFECTION

Three types of preparations contain uncooked, small and medium-sized, fish: (1) *Koi pla*, eaten soon after preparation; (2) Moderately fermented *pla som*, stored for a few days to weeks; and, (3) *Pla ra* extensively fermented, highly salted fish, stored for at least 2-3 mo^[2,11]. The consumption frequencies of *koi pla* in some communities every week was approximately 80%^[12]. In the northeasterners who have eaten *koi pla*, studies found the highest prevalence of liver fluke infection^[13,14]. The frequencies of *koi pla* consumption have declined and are generally confined to special social occasions, while other under-cooked fish preparations like *pla som* and other moderately preserved fish are generally eaten several times a week^[11]. *Pla ra* and *jaenbbong*, fully preserved fish, is an important staple and consumed daily by 60%-98% of northeasterners and lowland Laotians^[12,15]. At present, the patients still show that *Koi pla* is probably the most infective, followed by fish preserved for < 7 d, then *pla ra* and *jaenbbong*, in which viable metacercaria are rare^[11].

EPIDEMIOLOGY OF OPISTHORCHIASIS IN THAILAND

The Helminthiasis control program started in 1950 included opisthorchiasis control in some high risk areas^[16]. The main liver fluke control strategies comprise of three interrelated approaches, namely stool examinations and treatment of positive cases with praziquantel for eliminating human host reservoir; health education for a promotion of cooked fish consumption to prevent infection, and the improvement of hygienic defecation for the interruption of disease transmission^[16]. In Thailand, more than 7 million people were infected with *O. viverrini*, estimated by various investigators^[2,4,17,18]. Prevalence rates of *O. viverrini* in the northeast and the north were, 29.8% and 10.3% respectively^[19]. A health survey was carried out among residents of 33 villages under the Phitsanulok Irrigation Project Area, Nan River Basin, and northern Thailand. The prevalence of *O. viverrini* was 20%, the significant endemic diseases as potential health problems in this water resources development^[20]. A study of the prevalence and intensity of *O. viverrini* in relation to morbidity as determined by standard medical examination was carried out in Nong Ranya, a small village containing 309 people in northeastern Thailand. *O. viverrini* infection was determined with an overall prevalence of 94%, and reaching 100% prevalence in most age groups above the age of 10 years. Peak intensity in both males and females occurred at age 40 and above^[21].

In 1980-1981 the prevalence in the north, northeast, centre and south of Thailand was 5.59, 34.60, 6.34, and 0.01%, respectively, with an overall prevalence of 14% or

7 million people^[22]. Incidence, measured as the proportion of persons whose stools become positive within one year, was studied in endemic *O. viverrini*, in a northeastern Thai village over a two-year period. Incidence was higher in males than in females, especially in children under five years of age. It was at least 47% overall in the first year of the study, but declined to below 20% per year in the second^[23]. The prevalence and intensity of *O. viverrini* infection were investigated among 559 patients who were born in, and had lived all their lives in, either the rural or urban northeastern Thailand. 344 (79.4%) of 433 rural dwellers were infected compared with only 69 (54.8%) of 126 urban dwellers. Infection due to *O. viverrini* appears to be mainly a rural problem strongly associated with the habit and frequency of eating koi-pla^[24]. Tesana *et al* reported the prevalence of *O. viverrini* infection in the villages on the banks of rivers and those far from the rivers in Loei and Nong Khai Provinces, northeast Thailand. Most of the people examined in the present study were agriculturalists. The overall prevalence of *O. viverrini* infection was 41.3%. The prevalence of infection in males and in females in the villages far from the rivers were 52.6% and 51.7%, respectively, while the percent of people in the villages on the banks with infection were 27.9% and 21.7%, respectively. Prevalence of infection among the people residing far from the rivers was higher than those residing on the banks. This was observed despite the higher recording of raw fish consumption in villages on the banks. Infection level increased sharply in the age-group 6-10 years among people residing far from the rivers. High prevalence of infection was observed in age groups from 11 to 50 years^[25].

The patterns of infection with *O. viverrini* within a human community assessed by egg count were observed. A striking 81.5% of the total *Opisthorchis* population and 74% of the total egg output were expelled by the most heavily infected 10% of the humans sampled^[26]. Meanwhile, Sithithaworn *et al*^[27] investigated *O. viverrini* infection in 181 accident subjects in northeast Thailand. The prevalence increased rapidly with age and reached a plateau at 70%-80% in adults. The overall prevalence estimated by faecal examination was 69.2%, while that measured by worm recovery was 79.2%. In 1991, the survey of *O. viverrini* in 14 villages in Nakhon-Phanom province, Northeast, Thailand was conducted. Overall prevalence of *O. viverrini* infection was 66.4% in a total population of 2412 individuals. The prevalence was 18.5% in children under 5 years, 38.9% in those aged 5-9 years, and ranged from 64.9% to 82.2% in the age group above 10 years. The intensity of *O. viverrini* infection increased with age. The mean faecal egg output was highest in the 30-34 years age group and remained relatively constant in the older aged group. In all age groups the prevalence and intensity of infection in both men and women were similar. The population was divided according to the presence and intensity of infection as follows, 33% were uninfected, 59% had light infections (less than 1000 eggs per g of faeces; EPG), 7% had moderate infections (1000-10000 EPG), and 1% had heavy (greater than 10000 EPG)^[28]. Thereafter, Peng *et al*^[29] have been reported that all 1364 Thai labourers in Taiwan were examined for stool samples

and 18.0% were found to be infected, with *O. viverrini* at 7.0%. The prevalence was highest among the 21-25 age group (24.8%). The finding that parasitic infections are prevalent among Thai labourers demonstrates the need for control measures in foreign labourers in Taiwan. Meanwhile, stool samples from 93 Thais working in Israel were examined for the presence of parasites. The overall prevalence of infection by 1 or more species was 74%. *O. viverrini* and hookworm were the most prevalent parasites (51.6% and 44.1%, respectively)^[30]. In 1994, Radomyos *et al*^[31] examined *O. viverrini* infection in 681 residents from 16 provinces in northeast Thailand. The prevalence of *O. viverrini* in this group was 92.4%. In the same period, region wide assessments in 1994 were conducted. The prevalence of opisthorchiasis was 18.5% with a large variation in infection rate^[32].

In 1998, 431 residents from 16 provinces in northern Thailand who had previously been found positive for *O. viverrini* or *O. viverrini*-like eggs were given praziquantel 40 mg/kg. The stool was collected for 4 to 6 times and examined for adult worms. The prevalence of *O. viverrini* in this group was 11.6%^[33]. Waree *et al*^[34] survey the parasitic infection from 584 stool specimens in Noen Maprang, Phitsanulok Province during October 1999 to March 2000. It was found that the prevalence of *O. viverrini* infection was 10.78%. During October to November 2000, faecal samples were collected from study participants from 332 rural northeast Thais, *O. viverrini* was 14.2% and ranging from 8.6-19.4^[11]. While Wiwanikit *et al*^[35] reported the prevalence of *O. viverrini* was 8.7% (16 from 183 cases) in Sawasdee Village in the Nam Som District, Udonthani Province in northeastern Thailand in 2001. A cross-sectional study of the prevalence of intestinal parasitic infections at 8 schools in Bo Klau district and 4 schools in Chalerm Prakiet district, Nan Province, in January and February, 2001. A total of 1010 fecal samples were examined and found *O. viverrini* was 1.7%^[36]. Of 2213 Thai workers who visited the Out-patients Department of the King Chulalongkorn Memorial Hospital between September 2000 and January 2001 the prevalence of *O. viverrini* was 28.9%^[37]. Rhongbutsri *et al* reported the liver fluke infections, *O. viverrini* (11.1% from 395 samples), the most common in the old age group (51 years and up) in Ban Khok Yai Village, Khon Kaen Province, northeast Thailand^[38]. The nationwide survey was reported in 2001 by Jongsuksantigul and Imsomboon, the prevalence of opisthorchiasis in the north, northeast, central and south, 19.3%, 15.7%, 3.8% and 0% respectively^[16]. A small scale survey in Aranyaprathet District, Sa Kaeo Province, eastern Thailand, was conducted in January 2004. Of the 545 stool samples collected and examined, 261 (47.9%) contained small-sized eggs resembling *O. viverrini* eggs^[39]. Of 24723 participants in Khon Kaen Province of northeast Thailand, 18393 aged 35-69 years were tested for *O. viverrini* infection, by examining stools for the presence of eggs. The average crude prevalence of *O. viverrini* infection in the sample subjects was 24.5%, ranging from 2.1% to 70.8%^[40]. Recently, a total of 479 stool specimens were collected from rural communities of Ubon Ratchathani Province, Thailand. The prevalence of *O. viverrini* was 14.8%, while the same research group reported an epidemiological survey was conducted in Khon Kaen

Province involving 1124 stool samples using the modified Kato technique. The greatest frequency was *O. viverrini* at 32.0% while the second highest was *Sarocystis spp* at 8.0%^[41].

Report cases of opisthorchiasis per 100 000 populations by year, Thailand

The mortality rate was reported by the Bureau of Epidemiology, Department of Disease Control, Ministry of Public Health, Thailand during 2001-2006. Morbidity rate of opisthorchiasis cases decreased from 1.74 in 2001 to 0.79 in 2002. In 2003, the morbidity rate increased to 1.58, but then, the ratio slightly decreased from 1.33 in 2004 to 0.64 in 2005. In 2006, the morbidity rate slightly increased to 0.73 per 100 000 populations (Figure 1A).

Report cases of opisthorchiasis by month

During 2002-2006, the reported cases of opisthorchiasis by month were presented by the Bureau of Epidemiology, Department of Disease Control, Ministry of Public Health, Thailand. In 2002, the highest number of cases was in January, and the second was in March. Other months were a few cases of opisthorchiasis during the year. In 2003, the highest number of cases was reported, more than the other years from 2002 to 2006. The pattern of reported cases at each month, February was the highest and second was September. February in 2004 was the highest of reported cases followed by September and July. Meanwhile, in 2005 was found the highest opisthorchiasis cases in September, however, the second was February. Recently, in the reported cases February was higher than other months in 2006 (Figure 1B).

Report cases of opisthorchiasis by age-group, sex and occupation

Infection with *O. viverrini* begins at a very early age (0-4 age group, 0.64 per 100 000 population); the prevalence of infection rises rapidly with age up to adult hood and remains relatively high, thereafter, the relationship of prevalence to age being a function of "Koi pla" consumption. The intensity of infection (faecal egg output) in both males and females rises steadily in early life, reaches highest in the 55-64 years age group (2.21 per populations), the reported cases were found common in 35-44, 45-54, 55-64 and 65+, 1.48, 2.06, 2.21 and 1.43 per population, respectively (Figure 1C). This figure showed similarity with a previous study, however, Upatham *et al* reported the highest in the 35-44 years age^[14,21]. Loaharanu and Sornmani estimated the total direct cost of the infected work force (between the age of 15 and 60-year-old) in northeast Thailand to be Baht 2115 million per annum (wage loss = Baht 1620 million; direct cost of medical care = Baht 495 million)^[42]. Meanwhile, the more frequently reported cases were in males more than females, in a ratio of 1:1.11 (Figure 1D). The opisthorchiasis cases reported by occupation found that agriculture was the most highest of cases than other. It is very surprising that the student group was the third of reported cases during 2003-2006 (Figure 1E). This is a very serious public health problem, health education should be conducted through communication and education.

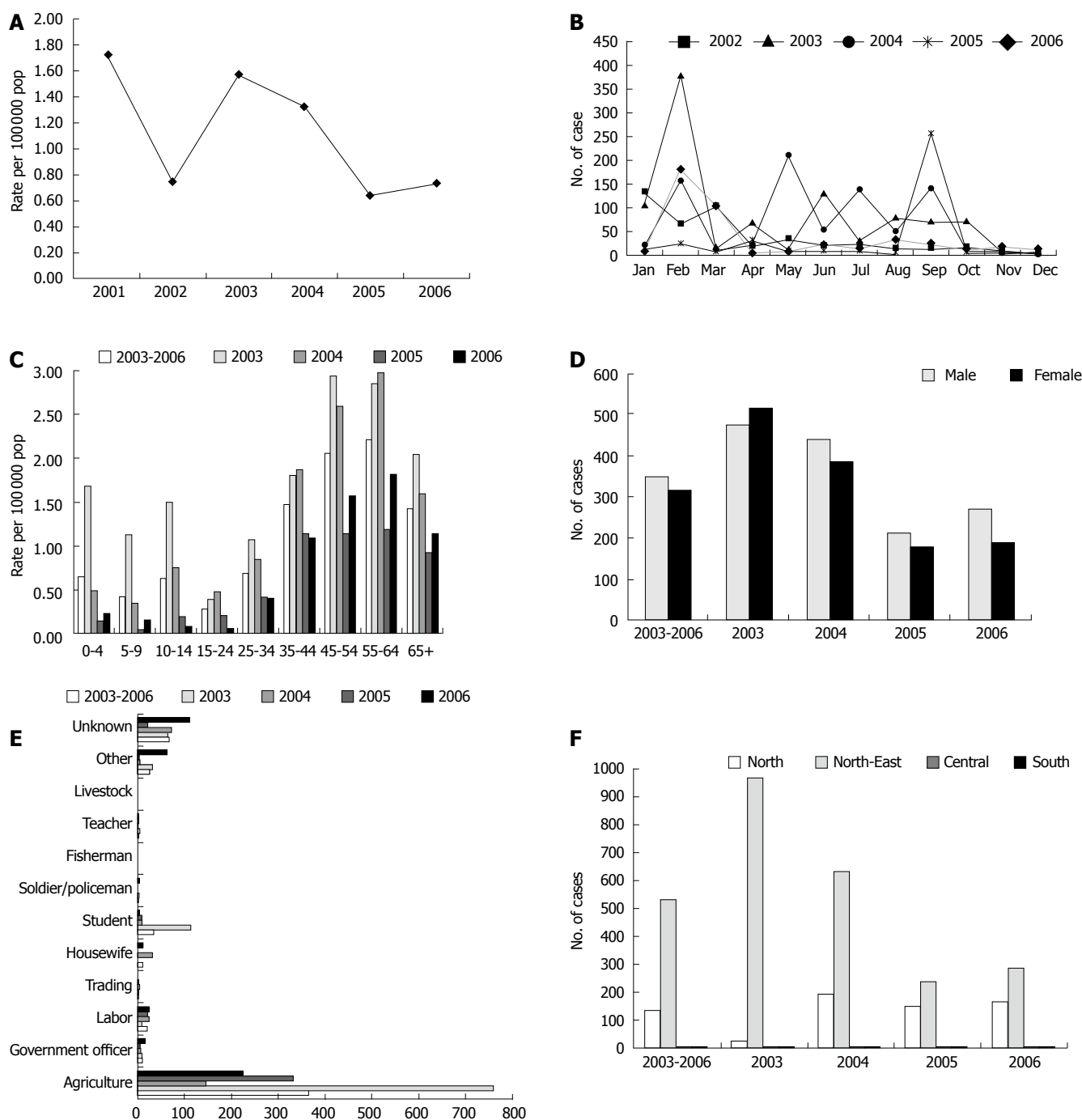


Figure 1 Reported cases of opisthorchiasis in Thailand^[43]. **A**: Reported cases of opisthorchiasis per 100 000 populations, 2001-2006; **B**: Reported cases of opisthorchiasis by month, 2002-2006; **C**: Reported cases of opisthorchiasis per 100 000 populations by age group, 2003-2006; **D**: Reported cases of opisthorchiasis by sex, 2003-2006; **E**: Reported cases of opisthorchiasis by occupation, 2003-2006; **F**: Reported cases of opisthorchiasis by region, 2003-2006.

Report cases of opisthorchiasis by region

Nationwide survey was reported by^[16]. The prevalence of *O. viverrini* infection in the north (19.3%), the northeast (15.7%), central (3.8%) and the south (0%) was recently reported. However, from 2003-2006, the reported cases of opisthorchiasis by region were presented by the Bureau of Epidemiology, Department of Disease Control, Ministry of Public Health, Thailand found that the northeast region was the highest of frequency than other regions. The second was the north, while the central region has shown a few opisthorchiasis cases. In the southern region, no cases were reported during 2003 to 2006 (Figure 1F). Although,

the reported cases of opisthorchiasis in the northern region were less than the northeast region in 2005-2006, the morbidity rate was higher than other regions. In 2006, the morbidity rate of opisthorchiasis cases in Thailand was 0.73 per population and in the north, northeast, central and south region was 1.45, 1.34, 0.00 and 0.00 respectively.

Report of cases of opisthorchiasis by province

It has also been shown that the prevalence and intensity of infection are greater among rural dwellers than their urban counterparts, an observation strongly associated with the habit and frequency of eating “*Koi pla*”^[24]. This tradition

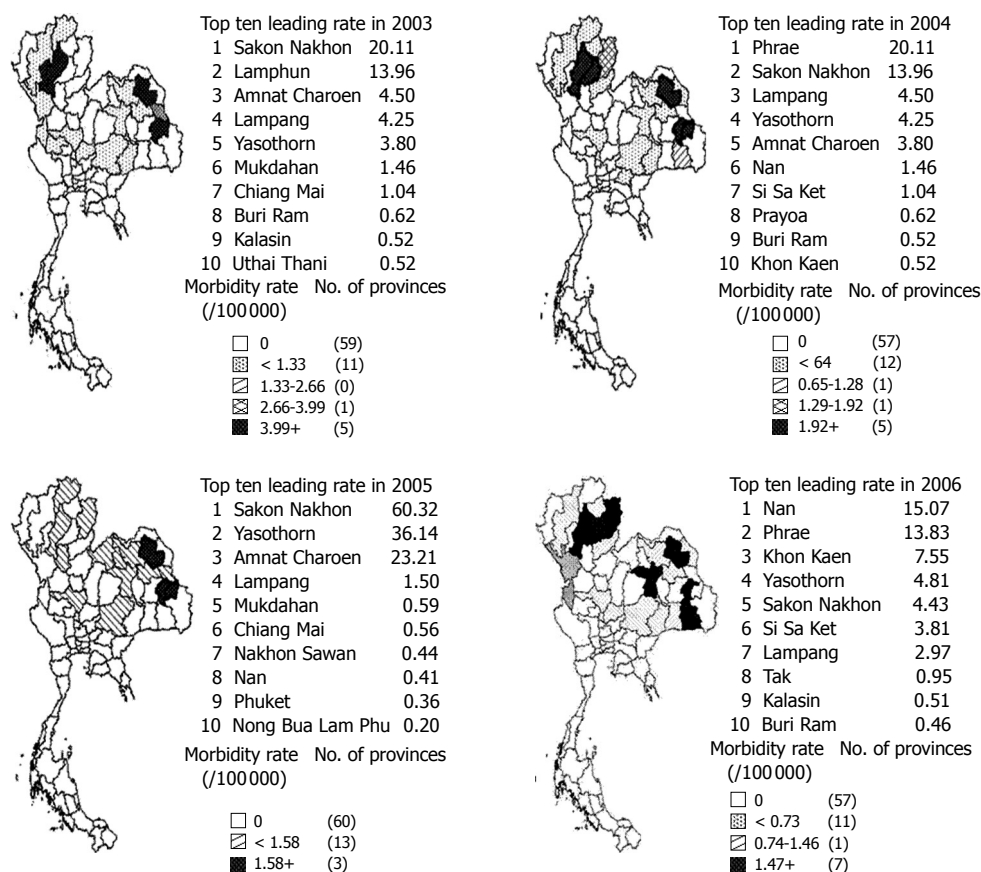


Figure 2 Reported cases of opisthorchiasis per 100 000 populations by province in Thailand, 2003-2006^[43].

is commonly found in the northeast and north region of Thailand, therefore, provinces located in 2 regions are also frequently reported with opisthorchiasis cases. The morbidity rate was identified into top ten leading rates during 2003-2006, Sakon Nakhon, Yasothon, Lamphun, Amnat Charoen, Lampang, Mukdahan, Chiang Mai, Buri Ram, Kalasin, Uthai Thani, Phrae, Nan, Si Sa Ket, Phayao, Khon Kaen, Chiang Rai, Nakhon Sawan, Phuket, Nong Bua Lam Phu, Phrae and Tak. From 2003-2006, Sakon Nakhon and Yasothon were the provinces that found the opisthorchiasis cases every year (Figure 2).

CONCLUSION

O. viverrini is a medically important food borne trematode in Thailand including some parts of Southeast Asia countries. The opisthorchiasis have been studied for more than 50 years; the infection is associated with cholangiocarcinoma and other hepatobiliary diseases. This review article emphasizes the passive surveillance data that was reviewed by the Bureau of Epidemiology, Department of Disease Control, Ministry of Public Health, Thailand. The epidemiology is found in some rural areas that the tradition of eating raw or uncooked fish products was the main reason for infection. The northeastern and northern regions of Thailand are still a major public problem in the high-risk areas.

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Gene therapy: Regulations, ethics and its practicalities in liver disease

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Abstract

Gene therapy is a new and promising approach which opens a new door to the treatment of human diseases. By direct transfer of genetic materials to the target cells, it could exert functions on the level of genes and molecules. It is hoped to be widely used in the treatment of liver disease, especially hepatic tumors by using different vectors encoding the aim gene for anti-tumor activity by activating primary and adaptive immunity, inhibiting oncogene and angiogenesis. Despite the huge curative potential shown in animal models and some pilot clinical trials, gene therapy has been under fierce discussion since its birth in academia and the public domain because of its unexpected side effects and ethical problems. There are other challenges arising from the technique itself like vector design, administration route test and standard protocol exploration. How well we respond will decide the fate of gene therapy clinical medical practice.

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Key words: Gene therapy; Liver disease; Hepatocarcinoma; Vector

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INTRODUCTION

Gene therapy is a newly developed approach which emerged at the end of the 20th century and aims to treat human diseases based on transfer of genetic materials to cells^[1]. It involves sets of recently developed technologies such as gene separation and purification, vector choice (viral and non-viral nature), transfer technique, *etc*^[2]. This approach develops quickly and has the potential to bring a new era to the treatment of human diseases, though the fierce debate and discussion on its ethics and practicalities in human beings have never stopped since its birth.

Like conventional therapy, gene therapy is under the regulation of the Nuremberg Code (1947) and the Declaration of Helsinki (1964) which established the principal research ethics concerning the vulnerability and interest of the patient as well as the benefit of independent review. However, gene therapy also raises specific ethical issues and public concerns about the three main fronts. Firstly, there exists the risk of deliberate alteration in the human germ line, which may change the inherited nature of human beings. Secondly, various new technologies continue to propel such debates. Taking utero gene therapy for example, it is necessary and feasible in technique, to intervene in some genetic disorders during fetal development, but concerns about the issues of safety, transgenerational risks to the germ line and questions about the fetal awareness, have hampered its development. Thirdly, the impact of adverse effects has kept ethical debate going on. In September 1999, a teenager volunteer, Jesse Gelsinger, died after receiving the adenoviral gene therapy vector for the treatment of the inherited condition "Ornithine Transcarbamylase Deficiency" at the Penn State University. Three years later, in October and December 2002, the Necker Hospital in Paris announced two boys under gene therapy for X-SCID developed a form of leukemia^[3]. Further evidence appeared in the same year, when Li Z *et al* reported that retroviral vectors can cause oncogenic transformation^[4]. Though we still do not know to what extent the risks described by these cases can be translated to other trials, it has up-regulated the public concern and led to the stringency of safety assessments and patient monitoring in clinical gene therapy trials.

Nevertheless, the increasing problems and difficulties never put off public focus and scientists' enthusiasm on gene therapy for it has incredible huge potential in the treatment of human diseases and what we have found is only the tip of the iceberg. In the following part, we will review the existing policy and regulations on gene therapy,

especially introduce the point of view from Chinese researchers, and then outline the general information on its application in liver disease and vector choice. Finally, we will focus on gene therapy for hepatic tumors.

POLICY, ETHICS AND REGULATION ON GENE THERAPY

In 1988, the European Medical Research Council first declared a formal stance against germline gene therapy, which was followed by the Council of Europe in 1991, for its violation of the basic human right to inherit a natural and unchanged genetic pattern from parents^[5]. Against this background, many individual countries have their own policies. Holland (1989) and the USA (1982) both postponed germline gene therapy due to ethical and technical barriers while Germany (1987) made it a criminal offence in a more rigorous policy^[5]. However, with the developments in biomedicine, ethical and policy analysis, the notion and practicalities of gene therapy have been under reevaluation. Some scientists have pointed out that “gene therapy” may inadequately describe the process and goals of germline alteration and should be replaced by the phrase “human germline genome modification” (HGLGM)^[6].

Unlike the ethical problem of germline gene therapy, somatic gene therapy attracts public controversy on its serious and unexpected side effects, which, in its utmost extent, have taken people's life away. Nevertheless, somatic gene therapy has been shown to be of great value in the treatment of different human diseases^[7], which makes a big challenge for scientists to control its advantages over disadvantages. Another related issue is the moral and risk evaluation of conducting gene therapy research in healthy volunteer subjects for there can be only long term potential risk and side effects with no exact benefits for patients. Anyway, most countries support the moral legitimacy of somatic-cell gene therapy for the cure of disease^[8].

Although gene therapy has the above mentioned risks when applied to human beings, its potential benefits could be released by our efforts. We have already done something to minimize the risks and maximize its treatment value in human diseases. Except for the legislation of general medicines, many nations have developed centralized but non-statutory device on gene therapy especially referring to the ethics. Under this condition, some specific national ethics committees and advisory boards like the USA Recombinant DNA Advisory Committee (RAC), the UK Gene Therapy Advisory committee (GTAC) and the Australian Gene Therapies Research Advisory Panel (GTRAP) were established. The aim of these regulation committees is to steer gene therapy towards the right direction.

It is interesting to note that a PubMed search of the term “ethics gene therapy” provides 1097 citations, and when narrowed to different Western countries there are always still many papers. However, when narrowed to “ethics gene therapy in China”, only two papers appear—one from UK, the other from Germany but none from China. In terms of language restriction and the large population, it is meaningful to introduce the general

regulations and ethics' consideration of gene therapy in China. After search through the Chinese biomedicine web database and Chinese scientific journals database, a total of 42 reviews were retrieved focusing on ethics of gene therapy. To sum up, currently there are no specific national ethics committees and advisory boards in China at different levels and comparing with Western countries, the legislation of gene therapy is still lagging behind, though three regulations including “the management of the safety of genetic engineering”, “the clinical and research control of human somatic gene therapy”, and “the management of human genetic resource” were established and all gene therapy-related activities are under their supervision. In academic arena, gene therapy also seems to be a grey area, where germline gene therapy is forbidden but somatic gene therapy and other therapies involving genetic engineering techniques are under intensive research. To our knowledge, the researches on gene therapy of hemophilia and mediterranean anemia are supported by Chinese government and some breakthroughs have been achieved. It seems that the awareness of and discussion on gene therapy at public level are less fierce than those in Western countries and actually, people are more interested in the beneficial and potential risks of transgenic food, which is closely related to their daily lives.

GENE THERAPY FOR LIVER DISEASE AND VECTOR CHOICE

The liver has vital functions in metabolism of lipids, carbohydrates and proteins while liver disease damages the synthesis of plasma proteins and coagulation factors, the clearance of lipids and toxins from serum, the secretion of bile to intestine, *etc*^[9]. Successful gene therapy needs relevant therapeutic gene, appropriate promoter and regulatory elements, effective vector to deliver the transgene into target cells. End-stage liver failure irrespective of its cause has a high morbidity and mortality while conventional medicine has little ability to promote recovery. However, successful gene therapy for liver disease has been achieved in animal models^[10]. Introducing the therapeutic gene through adenoviral vector has corrected hyperbilirubinemia in a Gunn rat model of Crigler-Najjar syndrome type I^[11,12]. Conlon TG *et al*^[13] found that intramuscular administration of recombinant adeno-associated virus (rAAV) vectors expressing short-chain acyl-CoA dehydrogenase (SCAD) could systemically correct the fatty acid oxidation disorder in SCAD-deficient mice. There are many kinds of liver disease which would benefit from gene therapy, though it still has a long way to go.

The successful gene therapy for liver disease largely depends on the development of gene delivery vector which could mainly be divided into viral and non-viral vectors. In the following, we will outline both sub-divisions.

Adenovirus is a double-stranded DNA virus which possesses a combination of features that make them highly suitable as vectors for expression of a heterologous gene^[14]. Adenoviral vectors are widely used in experimental gene therapy for cancers and also have a natural tropism

for the liver and a high efficiency for transferring non-dividing cells^[15]. The non-integrating feature makes it a two-edge sword, which, on the one hand, will decrease the risk of oncogenic side effects caused by gene integration and mutation, on the other hand, leads to transient expression in host cells. Recently, the so called high-capacity adenoviral (HC-Ad) vectors, lacking all the viral sequences except for the packaging signals, provides a prolonged transgene expression by escaping the host immune response^[16]. Goncalves MA *et al*^[17] have generated a hybrid gene transfer vehicle consisting of recombinant adeno-associated virus (AAV) replicative intermediates packaged in adenovirus (Ad) capsids for stable transduction of large DNA.

Retrovirus is a single stranded DNA virus which has the ability to integrate into the host cells. This characteristic increases the long term expression of the transferred gene in host cells but also results in potential side effects like leukemia caused by insertional mutagenesis. In order to decrease such risks, scientists have been exploring the molecular detail of genome packaging, retrovirus assembly and target site selection^[18,19]. Retroviral vectors have another shortcoming in not easily transferring cells like hepatocytes that do not proliferate actively under physiological conditions. However, the new development of human lentiviral vectors, allowing for the transduction of non-dividing cells and stable gene expression, has shed light on the way forward^[20].

Adeno-associated virus (AAV) is a non-pathogenic human parvovirus with the deletion of all viral genes except for ITR. This vector exhibits a number of properties, making it an excellent choice of CNS gene therapy^[21], for hemophilia^[22], lung disease^[23], retinal disease^[24], *etc.* Despite its low immunogenicity, toxicity, long-term transgene expression and ability to transduce dividing and non-dividing cells, it has also shown a limited capacity of accommodating foreign genes and to some extent, the oncogenic trait, due to gene integrating^[25,26]. There is still a lot of work to do on this specific vector.

There are also other viruses used as vectors like herpesvirus, baculovirus and the list is still expanding^[27]. However, the non-viral vector has its own advantages, which could avoid the problems caused by the viral vector such as endogenous viral recombination, oncogenic effects and unexpected immune response^[28]. Due to these traits, the non-viral vector has been rapidly developed and categorized into two groups: naked DNA delivery with a physical method like gene gun and electroporation or with chemical carriers such as polymer, peptide and lipid. Though various kinds of non-viral vector have appeared and new transfer techniques are emerging, it still has a long way to overcome its low transfection efficiency caused by extracellular and intracellular barriers and organ specificity^[29,30].

GENE THERAPY FOR HEPATIC TUMORS

Hepatic tumor ranks fifth in frequency worldwide among all malignancies and causes one million deaths annually^[31]. It is hard to cure when its progression precludes surgical resection and other conventional

techniques, like transarterial chemoembolization and systemic chemotherapy, are of less help because of their low efficacy and high complication rate. Transfer of therapeutic genes to the tumor or peritumor tissues has opened a new door to the treatment, and great efforts have been made to promote this approach at both preclinical and clinical levels. A large number of methods can be chosen for gene therapy, including activation of tumor suppressor genes, inhibition of oncogene and tumor angiogenesis, promoting specific gene sensitivity to drugs, transfer of oncolytic virus and stimulation of anti-tumor immunity^[32].

Interleukin (IL)-12 with a potent anti-tumor activity has been extensively studied during the past decades. IL-12 exerts its function as a bridge between innate and adaptive immune responses by inducing TH1 lymphocytes, inhibiting tumor angiogenesis, activating NK cells and cytotoxic T lymphocytes, and facilitating lymphocytes immigrating into the tumor tissues by up-regulating the expression of adhesion molecules on endothelial cells^[33,34]. However, this cytokine is toxic when administrated systematically either as a recombinant protein or as a naked DNA (encoding IL-12)^[35]. So what the scientist should do is to find the right way to express IL-12 constrained in local tumor tissue while minimize its systemic toxicity by decreasing the sera concentration.

As a reward for continuous effort, some breakthroughs have been made. Firstly, intra-tumor administration of recombinant adenovirus encoding IL-12 could eradicate neoplastic liver nodules in most of the animal models and increase long term survival. More surprisingly, treating one hepatic lesion also could lead to tumor elimination in a second non-treated hepatic lesion^[36]. This phenomenon may be explained by the strong hepatic tropism of adenovirus as it escapes from the injected nodule to the general circulation and then infects the whole liver. Secondly, based on the adenoviral vectors, some changes in vector design have been made to increase the transfection efficiency and decrease the systemic toxicity. Wang *et al*^[37] have generated a gutless adenoviral vector containing a mifepristone (RU486)-inducible system for liver-specific expression of human interleukin-12 (hIL-12) (GL-Ad/RUhIL-12), which allows a prolonged, regulateable, and tissue-specific transgene expression compared with normal adenoviral vectors. Waehler *et al*^[38] demonstrated that intra-tumor adenoviral IL-12 immunotherapy can substantially improve its anti-tumor efficacy and safety profile when a fusion protein of two subunits of IL-12 (scIL-12) is expressed in an adenoviral vector. Dickerson *et al*^[39] have engineered a fusion protein of IL-12 linking the vascular homing peptide CDCRGDCFC to directly target the tumor neovasculature. Significant enhancement of antiangiogenic effect, augmentation of anti-tumor activity, and decreased IL-12 toxicity were observed. Thirdly, different administration ways have been developed and tested. Except for the above mentioned intra-tumor and peri-tumor injection, adenovirus encoding IL-12 given by intra-hepatic arterial route^[36] and portal vein route^[40] has been also shown to significantly reduce tumor burden and prolong survival. Fourthly, though IL-12-based gene therapy has pivotal anti-tumor effects, the

toxicity caused by inducing interferon gamma production has more or less hampered its application. To solve this problem, scientists are trying to combine IL-12 with other chemokines such as IP-10, to attract immune effector cells to tumors through IP-10 production and to activate attracted lymphocytes with IL-12^[41]. We can also generate IL-12 secreting dendritic cells (DCs) by infecting them with adenovirus encoding IL-12 *in vitro* and then injecting these engineered DCs into the tumor^[42]. This approach has proved extremely effective on liver tumor metastases from colorectal carcinoma^[43]. Finally, adenovirus encoding IL-12 also has the ability to induce anti-tumor effects on liver neoplasms metastasized from other organ tumors^[44,45]. This effect may be mediated by nonlymphocyte effector cells including macrophages and neutrophils and involve anti-angiogenic chemokines^[46].

Besides IL-12 gene therapy for hepatic tumor, there are other methods of gene therapy under exploration. Since p53 is mutated in approximately 50% of human tumors and has an important role in the genesis or progression of hepatocellular cancers, we could use gene replacement therapy of p53 for tumors^[47]. It was reported that adenovirus encoding CD40 ligand could induce protective and curative anti-tumor immunity^[48]. There is evidence that combination of adenovirus encoding CD40 ligand and naïve dendritic cells can decrease the amount of CD40 ligand while maintaining normal anti-tumor effect levels^[49]. Silencing of oncogene or other genes by RNA interference (RNAi) offers a promising approach to the treatment of hepatic tumors^[50]. We can also use isolated hepatic perfusion (IHP) to increase the adenoviral vector transfection efficiency and decrease the systemic toxicity^[51]. Transfer of suicide gene into tumor tissue is another way of gene therapy. Terazaki *et al*^[52] demonstrated that optimal therapeutic expression level of a suicide gene is a novel concept and a promising method.

Although gene therapy for hepatic tumor has been proved effective in most animals, there is still a lack of information about the efficacy and safety of those treatments in humans. Therapeutic gene, dosage and route of administration, type of vector and tumor itself are all complex ingredients and need careful consideration. Gene therapy is mainly used at the moment as a supplement to conventional treatment, and many trials are carried out in patients with advanced tumors. Thus, shortage of data about early cancer may underestimate the real effect of gene therapy on tumors. However, with the booming of phase I and II clinical trials, we will get enough information for analysis and estimation of gene therapy and this treatment method has a bright future.

CONCLUSION

Gene therapy is a new and powerful tool to correct inherited disorders and treat human diseases. To some extent, this approach can be considered a revolution in the history of medicine for it deepens our vision on the nature of diseases and broadens our methods of treatment focusing on the level of genes and molecules. It can be used not only in the treatment of hepatic tumor which we have described in detail but also in other diseases once

there are any changes at gene level. Though animal models and some pilot clinical trials have shown a convincing future, gene therapy is still at its beginning and there is a lot of work to be done. Referring to the techniques, we should improve the transduction efficiency of vectors, increase the duration of therapeutic gene expression, decrease the unexpected toxicity and side effects, test and polish the routes of drug administration, *etc.* There is also likely a challenge of ethical issues. We should remember that technique itself is innocent, whether it is an evil or a virtue depends on the users. So it is our obligation to establish consummate regulations and policies of gene therapy making it serve human beings. Another problem is how to make gene therapy affordable to ordinary patients. As a new approach, gene therapy is much more expensive than conventional therapy, which is a hurdle for its wider use. How to balance the relationship between gene therapy and conventional therapy is very important. Gene therapy has been used to complement conventional therapy for end-stage diseases that cannot be cured with the latter. With the progress in gene therapy, more and more diseases can be cured at their early stage when conventional therapy is still useful.

In conclusion, despite the difficulties and obstacles, gene therapy has the potential to become a cornerstone of modern medicine.

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COLORECTAL CANCER

Inhibition of CXCR4 activity with AMD3100 decreases invasion of human colorectal cancer cells *in vitro*

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cancer cell line SW480 through down-regulation of VEGF and MMP-9 expression.

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Abstract

AIM: To investigate the effect and mechanism of blockade of the CXC chemokine receptor-4 (CXCR4) signaling pathway by AMD3100, a small non-peptide CXCR4 inhibitor, on invasion and metastasis of colorectal cancer cells *in vitro*.

METHODS: Human colorectal cancer cell line SW480 was treated with AMD3100 at different final concentrations. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay was used to detect the effect of AMD3100 on cell proliferation. The invasion ability of SW480 cells was determined by cell invasion assay kit. In the presence of AMD3100, the CXCL12-mediated migratory response of SW480 cells was tested by classical chemotaxis assays. RT-PCR analysis and Western blotting were used to detect the expression of vascular endothelial growth factor (VEGF), matrix metalloproteinase-2 (MMP-2) and -9 (MMP-9) in SW480 cells.

RESULTS: Cell viability was significantly suppressed by AMD3100 in a dose-dependent manner. AMD3100 (100 and 1000 ng/mL) significantly inhibited the invasion ability of SW480 cells. Treatment with AMD3100 markedly reduced the expression of VEGF and MMP-9 but not MMP-2 in SW480 cells.

CONCLUSION: The CXCL12/CXCR4 system is an important mediator of proliferation and invasion of CXCR4-expressing colorectal cancer cells. AMD3100 inhibited invasion and metastasis activity of the colorectal

INTRODUCTION

Stromal cell-derived factor-1 (SDF-1 or CXCL12) and its unique receptor CXC chemokine receptor-4 (CXCR4) have prominent roles in invasion and metastasis of a diverse number of cancers. The interaction between SDF-1 and CXCR4 has been shown to direct tumor cells to organ sites with high levels of SDF-1 expression, which suggests this molecular pair plays a key role in chemotaxis and homing of metastatic cells. Convincing evidence indicates elevated CXCR4 expression in primary tumors is associated with lymph node metastasis in breasts^[1-4], head and neck^[5], and colon^[6]. Furthermore, CXCR4 expression is associated with intraperitoneal carcinomatosis of ovarian^[7] and gastric^[8] cancer. High or persistent expression of CXCR4 has been associated with poor prognosis in osteosarcoma^[9], epithelial ovarian cancer^[10], colon cancer^[6,11,12], esophageal carcinoma^[13], and melanoma^[14]. Recently, intensive research has shown that binding of CXCL12 to CXCR4 plays a role in tumor angiogenesis by influencing the secretion of vascular endothelial growth factor (VEGF)^[15] and increasing invasion associated with matrix metalloproteinase (MMP)-9 activation^[16]. Therefore, interruption of the interaction between CXCR4 and SDF-1 has received considerable attention since it may provide a means of inhibiting the metastatic process.

AMD3100, a bicyclam molecule, has been identified as a specific inhibitor of CXCR4. It had originally been developed as an inhibitor of T-tropic human

immunodeficiency virus (HIV) infectivity and has been used to block HIV infection of T-tropic, X4-using, virus and induce X4 transformation to R5, which validate CXCR4 as a target for HIV therapy in clinical trials. Furthermore, AMD3100 has also been demonstrated to be an effective mobilizer of hematopoietic stem cells in both healthy volunteers and multiple myeloma and non-Hodgkin's lymphoma patients^[17,18]. In recent studies, anti-CXCR4 treatment has suppressed primary tumor growth by inhibiting tumor angiogenesis, and has prevented lung metastasis of squamous cell carcinoma of the head and neck (SCCHN) in animal models^[19]. Treatment of animals with AMD3100 resulted in decreased activation of the mitogen-activated protein kinase and AKT pathways in xenograft brain tumors, which are pathways downstream of CXCR4 that promote survival, proliferation and migration^[20].

However, the influence of various concentrations of AMD3100 on invasion and proliferation in human colorectal cancer cell lines has not been reported. The present study showed that inhibition of CXCR4, through using non-peptide small molecule AMD3100, had significant antitumor activity, which represents a novel strategy for targeting highly metastatic colorectal cancer cells.

MATERIALS AND METHODS

Cells

Human colorectal cancer (CRC) cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Tumor cells were maintained in RPMI 1640, supplemented with 10% fetal calf serum, 100 U/mL penicillin, and 100 µg/mL streptomycin in a humidified incubator with an atmosphere of 5% CO₂, 95% air at 37°C. The specific chemokine receptor CXCR4 antagonist, AMD3100 (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in sterile PBS (GIBCO, Carlsbad, CA, USA) as a × 1000 stock solution, and then diluted with the culture medium for experiments.

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay

Cell viability was determined by MTT assay. Briefly, exponentially growing CRC cells were seeded in 96-well culture plates in serum-free medium at an optimal density. After 24 h incubation, either PBS or the indicated dose of AMD3100 was added for 2 h incubation in eight parallel holes. Then, CXCL12 (Peprotech, Rocky Hill, NJ, USA) was added daily at 20 ng/mL. MTT assays (Beyotime, Haimen, China) were performed after 24, 48 and 72 h of AMD3100 treatment. Absorbance was measured at 590 nm. The results were calculated as mean values of eight wells per treatment group.

Chemotaxis and invasion assays

Cell lines were assessed for migration utilizing a Boyden chamber chemotaxis assay. Chambers with 8 mm pore filters (HTS Transwell-24 System; Corning, Acton, MA, USA) were used. CXCL12 was added to the lower wells with 0.5% fetal bovine serum medium. Selected cells were

treated with PBS or AMD3100 15 min prior to assay performance. After 3 h incubation at 37°C, cells within the inserts were removed from the upper surface of the membrane using a moist cotton-tipped swab. Migratory cells on the lower surface of the membrane, which had migrated through the polycarbonate membrane, were fixed in 100% ethanol, washed with phosphate buffer solution, air dried and stained with crystal violet for 30 min, then rinsed several times with distilled water. Migration was quantitated by dissolving stained cells in 10% acetic acid, and an equal amount of the dye/solution mixture was transferred onto a 96-well plate for colorimetric reading of *A*₅₉₀. Invasion assay was performed using Cell Invasion Kit (Chemicon, Temecula, CA, USA). The inserts had an 8-µm pore size polycarbonate membrane with a precoated thin layer of basement membrane matrix (ECMatrix, Temecula, CA, USA). After 24 h incubation, invasive cells on the lower surface of the membrane were stained and calculated as previous described. Assays were performed in triplicate.

Western blotting

Whole-cell protein and nuclear protein extracts from SW480 CRC cells were prepared with Cell lysis buffer for Western and IP (Beyotime, Haimen, China) and Nuclear Extract Kit (Active Motif, Carlsbad, CA, USA), respectively, according to the manufacturers' instructions. Protein concentrations were determined using an assay kit (Bio-Rad, Hercules, CA, USA). Lysates containing 100 µg protein were mixed with loading buffer with 5% β-mercaptoethanol, and heated for 5 min at 100°C. Samples were separated by SDS-PAGE and transferred onto nitrocellulose membranes by semidry blotting. Membranes were incubated in TBS buffer for 1 h at room temperature, followed by hybridization with anti-CXCR4 antibody (Chemicon, Temecula, CA, USA; 1:1000 dilution), anti-MMP-9, anti-MMP-2 antibody, anti-VEGF antibody (Boster, Wuhan, China; 1:500 dilution) at 4°C overnight. After three washes in TBS/0.1% Tween 20, the membranes underwent hybridization with a horseradish peroxidase-conjugated secondary antibody, rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, CA, USA; 1:5000 dilution) for 1 h at room temperature. After three washes in TBS/0.1% Tween 20, signals were detected by chemiluminescence using the Western blotting luminol reagent (Santa Cruz Biotechnology).

RT-PCR

Total RNA extraction from CRC cells was performed with Trizol Reagent (Invitrogen, Carlsbad, CA, USA). Then, 2 µg total RNA was reverse transcribed with the First Strand cDNA Synthesis Kit (Takara, Japan). Subsequently, 2 µL cDNA product was subjected to PCR amplification with Taq DNA polymerase (Takara, Japan) on a thermal cycler using the following primers. The Random 9 mers primers for CXCR4, MMP-2, MMP-9, VEGF and β-actin were constructed on the basis of published sequences. The PCR primers used to detect each factor were as follows: CXCR4, sense strand 5'-GGAGGGGATCAGTATATAC A-3'; antisense strand 5'-GAAGATGATGGAGTAGAT

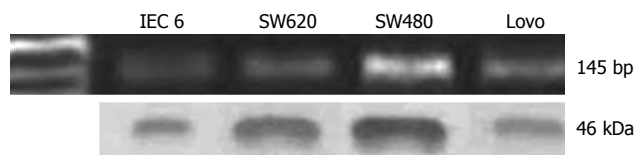


Figure 1 Expression of CXCR4 in intestinal epithelial cells. Expression of CXCR4 in highly metastatic CRC cell line SW480, using RT-PCR analysis of mRNA, and Western blotting of CXCR4 protein levels.

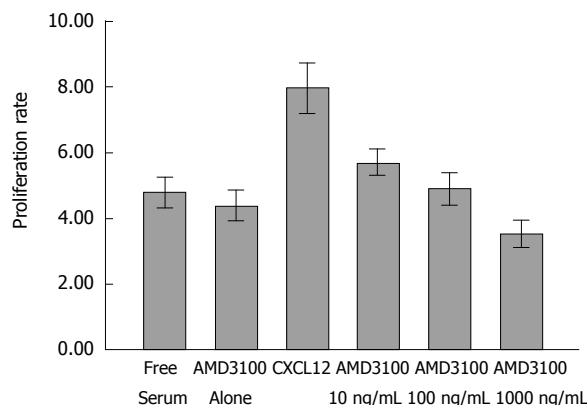


Figure 2 Effect of AMD3100 on viability of CRC SW480 cells. After 24 h incubation, cells growing in 96-well plates were treated with AMD3100 for 2 h. CXCL12 was added at 20 ng/mL per day, and the MTT assay revealed that in serum-free medium or the absence of CXCL12, AMD3100-induced inhibition was relatively weak. CXCL12-induced cell proliferation was significantly suppressed by 100 and 1000 ng/mL AMD3100 in SW480 cells. Cell viability was not significantly affected by 10 ng/mL AMD3100 (compared to the unstimulated group). Data are mean \pm SD of eight wells after 3 d incubation. Bars indicate mean \pm SD of triplicate experiments.

GG-3' (145 bp)^[12]; *MMP-2*, sense strand 5'-GTGCTGAA GGACACACTAAAGA-AGA-3'; antisense strand 5'-TTGCCATCCTTCTCAAAGTTGTAGG-3', (605 bp)^[21]; *VEGF*, sense strand 5'-CCTGGTGGACATCTTCCAGG AGTACC-3'; antisense strand 5'-GAAGCTCATCTCTC CTATGTGCTGGC-3', (196 bp)^[22]; *MMP-9*, sense strand 5'-TCCCT-GGAGACCTGAGAACC-3'; antisense strand 5'-GTCGTCGGTGTCTGTAGT-TGG-3' (704 bp)^[23] and β -actin, sense strand 5'-ATCTGGCACCA CACCTTCTACAA-TGAGCTGCG-3'; antisense strand 5'-CGTCATACTCCTGCTTGCTGATCCACATCTG-C-3' (838 bp)^[24]. The PCR conditions were as follows: One cycle of denaturing at 94°C for 2 min, followed by 30-35 cycles amplification, 56°C for 45 s and 72°C for 2 min. The PCR products were loaded onto 2% agarose gels and visualized with ethidium bromide under UV light. The ratio value of each group and the GAPDH group was taken as the relative value.

Statistical analysis

Numeric data are presented as mean \pm SD of three experiments. The paired student's *t* test or one-way ANOVA was used for comparing the differences between groups. Statistical significance was assigned if $P < 0.05$. Analyses were performed using SPSS 13.0 (SPSS, Chicago, IL, USA).

Table 1 Effect of AMD3100 on invasion of CRC cells (mean \pm SD)

Group	A ₅₉₀ value	Inhibition rate (%)
Control	0.703 \pm 0.016	
AMD3100		
10 ng/mL	0.656 \pm 0.062	7.57 \pm 7.21
100 ng/mL	0.505 \pm 0.091 ^a	28.43 \pm 12.86 ^a
1000 ng/mL	0.158 \pm 0.0325 ^b	77.23 \pm 4.26 ^b

^a $P < 0.05$, ^b $P < 0.01$, vs the control group ($n = 3$).

RESULTS

Expression of CXCR4 in intestinal epithelial cells

In our previous study, we analyzed CXCR4 expression in several cell lines (SW480, SW620, Lovo and IEC-6). In particular, our data showed lymph-node-metastasis-derived cell line SW480 expressed CXCR4 at a high level, by using RT-PCR and Western blotting (Figure 1). We also examined mRNA expression of *CXCL12*, the ligand of CXCR4, by RT-PCR. We found no expression of *CXCL12* mRNA in any of the colorectal cancer cell lines (data not shown).

Effect of AMD3100 on viability of CRC cells SW480

In our previous studies, we found no expression of *CXCL12* mRNA in any of the CRC cancer cell lines. After 3 d incubation, *CXCL12* greatly enhanced SW480 cells viability in the absence of serum (Figure 2). The enhancing effect of *CXCL12* on cell proliferation was strongly inhibited by treatment with different doses of AMD3100. In a dose-dependent fashion, the proliferation rate was reduced to 6.10 ± 0.13 , 4.49 ± 0.22 , 3.58 ± 0.13 respectively ($P < 0.05$). The effect of 100 and 1000 ng/mL AMD3100 was statistically significant ($P < 0.01$, $n = 8$) compared to that of the CXCL12 group (7.97 ± 0.811). Although a decrease in proliferation was also observed in the AMD3100 alone group compared to the serum-free cells (vehicle-treated cells), the inhibition rate was not significantly different, probably due to a specific effect of blocking CXCL12-CXCR4 interaction. The assay also revealed that, in 24 h, there was no significant difference in viability in any of the groups. Therefore, the cell invasion assay was performed at 24 h to remove its influence on cell viability.

Effect of AMD3100 on invasion of CRC cells

To evaluate the effects of inhibition of CXCL12-CXCR4 interaction on CRC invasion, we performed an *in vitro* invasion assay using AMD3100. After 24 h incubation, AMD3100 markedly reduced invasion of SW480 cells at concentrations of 100 and 1000 ng/mL (Table 1), by 28.43% ($P < 0.05$) and 77.23% ($P < 0.01$), respectively.

Effect of AMD3100 on chemotactic migration of CRC cells

The effect of AMD3100 on inhibiting CXCL12-induced migration of CRC cells was estimated by a classical chemotaxis assay. The selected CXCR4-positive cell line, SW480, did migrate in response to CXCL12 in a classical

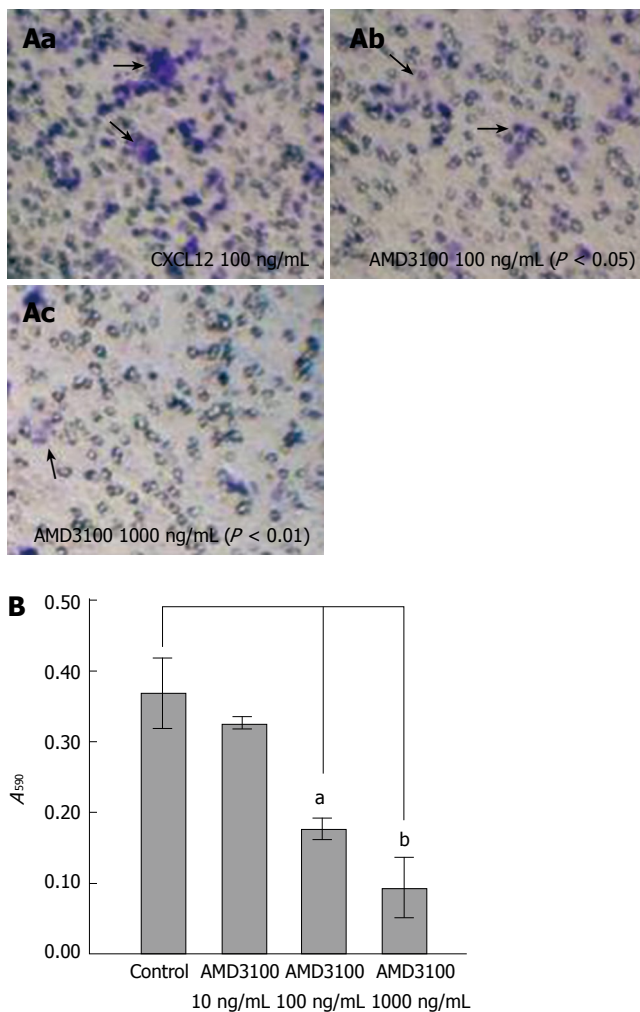


Figure 3 A: Effect of AMD3100 on chemotactic migration of CRC cells. The chemotaxis assay indicated that AMD3100 significantly inhibited the CXCL12-mediated migration of SW480 cells at final concentrations of 100 and 1000 ng/mL. The blue-stained cells are those that migrated through the polycarbonate membrane to the lower surface of the membrane (a-c); **B:** CXCL12 inhibited migration of SW480 cells in a dose-dependent manner. Bars indicate mean \pm SD of triplicate experiments. ^a $P < 0.05$, ^b $P < 0.01$.

chemotaxis assay, with an optimal response at 100 ng/mL. After AMD3100 treatment, chemotactic activity of SW480 cells was reduced in a dose-dependent manner (Figure 3B). The inhibition rate with AMD3100 at 10, 100 and 1000 ng/mL was 5.24%, 47.27% and 62.37%, respectively. The latter two achieved a significant difference compare to the control group (a, b and c in Figure 3A).

Effect of AMD3100 on expression of MMP-2, MMP-9 and VEGF in SW480 cells

The CXCL12-CXCR4 axis contributes to invasion and specific organ metastasis through regulation of its target genes, which have recently been shown to be *VEGF* and *MMPs*^[7,16,25,26]. Therefore, we detected the effect of AMD3100 on expression of VEGF, MMP-2 and MMP-9. As shown in Figure 4, 24 h incubation with AMD3100 reduced MMP-9 and VEGF protein expression in a dose-dependent manner in SW480 cells. RT-PCR demonstrated that the expression of *MMP-9* but not *MMP-2* and *VEGF* mRNAs in SW480 cells was significantly downregulated by

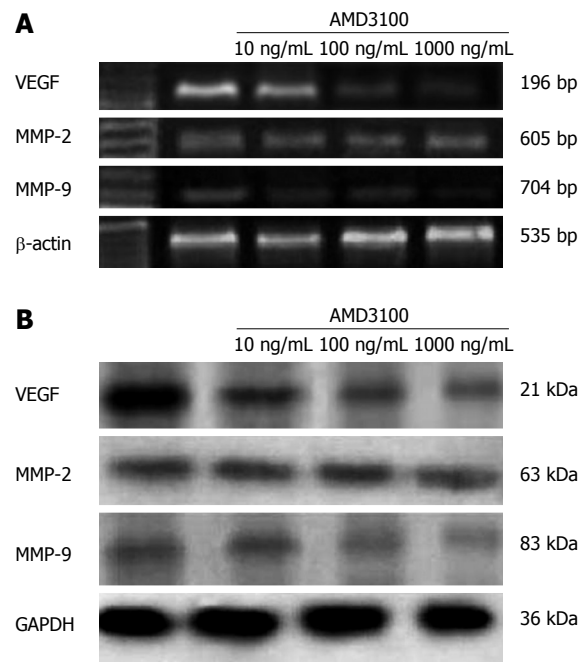


Figure 4 A: Effect of AMD3100 on expression of MMP-2, MMP-9 and VEGF in SW480 cells. Protein samples extracted from SW480 cells treated for 26 h with AMD3100 were subjected to Western blotting for MMP-2, MMP-9, VEGF and GAPDH proteins. AMD3100 significantly decreased MMP-9 and VEGF protein expression in SW480 cells in a dose-dependent manner. The level of GAPDH expression was determined as a control for equivalent protein loading; **B:** RNA samples extracted from SW480 cells treated for 26 h with AMD3100 were subjected to RT-PCR for *MMP-2*, *MMP-9*, *VEGF* and β -actin. AMD3100 also significantly decreased expression of *MMP-9* and *VEGF* mRNAs in SW480 cells, and the inhibitory effect was dose-dependent. RT-PCR for β -actin was performed in parallel to show an equal amount of total RNA in the sample.

100 and 1000 ng/mL AMD3100. Densitometric analysis revealed the relative expression decreased to $17.58\% \pm 3.79\%$ for *MMP-9*, and $39.44\% \pm 3.07\%$ for *VEGF*, as compared to the controls ($P < 0.05$).

DISCUSSION

A growing body of literature has indicated CXCR4 is important in a variety of cancers, and more specifically, that this receptor can be a propitious target in treating cancer. In experimental systems, convincing evidence has shown that selective inhibition of CXCR4 suppresses CXCL12-induced migration of cancer cells, invasion, neoangiogenesis and metastases. Neutralizing the interactions of CXCL12 and CXCR4 by monoclonal antibody significantly impairs metastasis of breast cancer cells to regional lymph nodes and lungs^[27]. Human breast tumor growth can be delayed by inhibiting CXCR4 with siRNA^[28]. Similarly, CXCR4 antagonists, T140 analogs, inhibit SDF-1-induced migration of human breast cancer, leukemia and endothelial cells *in vitro*, in a manner relevant to tumor spread and angiogenesis^[29]. The CXCR4 inhibitor RCP168 partially abrogates the protective effect of stromal co-culture system and greatly enhances chemotherapy-induced apoptosis in chronic lymphocytic leukemia cells^[30]. Redjal *et al* have demonstrated the ability of AMD3100 to reduce the activation of extracellular signal-regulated

kinases 1 and 2 and Akt, all of which are pathways downstream of CXCR4 that promote cell survival, proliferation and migration. Moreover, *in vivo*, combining sub-therapeutic doses of AMD3100 with a conventional cytotoxic agent in an order-dependent manner produces synergistic effects, which result from both a decrease in proliferation and an increase in apoptosis of tumor cells^[31].

Our previous studies have shown a significant association between CXCR4 expression and lymph nodal status and increased risk for metastasis in colorectal carcinoma. These initial observations prompted us to further question whether modulation of CXCR4 affects the invasive ability of CRC cells. In the present study, we demonstrated that AMD3100, a CXCR4-specific inhibitor, strongly suppressed CXCL12-induced activity and invasion of the SW480 cell line, which expressed CXCR4 at a high level. Recently, researchers have found that CXCL12-CXCR4 signaling directly regulates MMP-9 expression in CRC cell lines^[16]. In our present study, the use of AMD3100 markedly reduced the mRNA and protein expression of MMP-9 but not MMP-2 in SW480 cells, through blocking the CXCL12-CXCR4 signaling pathway. This suggests that inhibiting SDF-1/CXCR4 interaction may have a variety of therapeutic benefits in CRC patients. Meanwhile, we also observed a directional migration of CRC cells depending on the presence of extracellular matrix (ECM) proteins. AMD3100 significantly inhibited CXCL12-induced migratory activity and ECM-dependent direct invasive ability.

Recent studies have also shown VEGF expression is associated with poor survival and prognosis in CRC, and that increased VEGF expression is correlated with increased microvessel density, local invasion, liver metastasis, and early recurrence after curative resection^[20,32]. In the present study, we also found that AMD3100 significantly decreased mRNA and protein expression of VEGF in SW480 cells, which suggests that drug treatment may not only block CXCL12-induced angiogenesis, but also impair formation of microvasculature in metastasis stimulated by VEGF.

In serum-free medium, adding CXCL12 may significantly increase proliferation activity of tumor cells. This kind of protective microenvironment generally exists in various solid tumors, such as CXCR4-positive renal cancer and intracranial glioblastoma, which functionally improves cancer-cell viability, anti-apoptotic survival and chemoresistance^[29]. Production of CXCL12 in tumor stroma may keep cancer cells tolerant to hypoxic or poor nutritional conditions. On the other hand, hypoxia-induced CXCR4 expression may activate downstream signal transduction, and subsequently regulate cancer cell biological behavior to adapt to hypoxia. In our study, we tested in the presence of CXCL12, the effect of various concentrations of AMD3100 on cell viability. Interestingly, although the treatment failed to decrease the number of cancer cells in serum-free medium, AMD3100 abrogated CXCL12 stimulation during cell proliferation in a dose-dependent manner. Additionally, AMD3100 alone did not significantly affect cell proliferation compared with the unstimulated group, which suggests no autocrine growth stimulatory loops exist in this cell line.

In conclusion, CXCL12-CXCR4 signals are responsible for the invasion and metastasis of human CRC *via*

regulation of cell viability and the expression of genes related to proteolysis and angiogenesis. Blocking the CXCL12-CXCR4 pathway may provide a novel means of preventing the invasion and metastasis of CRC.

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COMMENTS

Background

CXC chemokine receptor-4 (CXCR4) is by far the most common chemokine receptor overexpressed in human cancer cells. High-level expression of CXCR4 in various primary cancers is significantly associated with poor prognosis and extent of metastasis. Targeting CXCR4, therefore, is now regarded as a novel and efficient strategy for treating human cancer metastases. There are two categories of therapeutic strategy for targeting CXCR4, anti-CXCR4 monoclonal antibody and specific small molecular CXCR4 antagonists such as AMD3100, T140, ALX40-4C and CTCE-9908. Each of these inhibits CXCR4 via different mechanisms.

Research frontiers

Specific small-molecule CXCR4 antagonists such as AMD3100 may play an important role in the treatment of HIV infections, and many other pathological processes that are dependent on CXCL12-CXCR4 interactions (e.g. rheumatoid arthritis, asthma and cancer metastasis). Treatment of animals with AMD3100 results in decreased activation of the mitogen-activated protein kinase and AKT pathways in xenograft brain tumors. Administration of AMD3100 and 1, 3-bis (2-chloroethyl)-1-nitrosourea (BCNU) in an order-dependent manner produces synergistic cytotoxicity, which results from both a decrease in proliferation and an increase in apoptosis of glioblastoma cells.

Innovations and breakthroughs

We have demonstrated that CXCL12-induced activity and invasion of CRC cells were markedly suppressed by blocking the CXCL12-CXCR4 signaling pathway with AMD3100, a small non-peptide inhibitors of CXCR4. These findings strongly suggest CXCR4 plays an important role in CRC progression and may provide a novel and effective molecular target for treatment of CRC.

Applications

Our study showed that blockade of the CXCL12-CXCR4 axis affects the viability, invasion and migration of CRC cells by using various doses of AMD3100. The data also demonstrated the inhibitory effect was dose-dependent. It may be a good start to move from elucidation of the mechanism of action of chemokine receptors in cancer metastasis to development of novel therapeutic targets based on the CXCL12-CXCR4 axis.

Terminology

Chemokines are small secreted peptides that control adhesion and transendothelial migration of leukocytes, especially during immune and inflammatory reactions. They are divided into four subfamilies: CC, CXC, C and CX3C based on the position of their NH₂-terminal cysteine residues, and bind to seven transmembrane domain G protein-coupled receptors. CXCL12, also known as SDF-1, belongs to the CXC chemokine family and CXCR4 is the only known physiological receptor for SDF-1.

Peer review

This is a very interesting study. The authors showed inhibition of CRC cell viability and metastasis by blocking CXCL12-CXCR4 interaction. Although this does not necessarily predict a positive result in human trials, the accumulation of these data may provide additional clues regarding some unanswered questions.

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BASIC RESEARCH

Munc18/SNARE proteins' regulation of exocytosis in guinea pig duodenal Brunner's gland acini

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Abstract

AIM: To examine the molecular mechanism of exocytosis in the Brunner's gland acinar cell.

METHODS: We used a submucosal preparation of guinea pig duodenal Brunner's gland acini to visualize the dilation of the ductal lumen in response to cholinergic stimulus. We correlated this to electron microscopy to determine the extent of exocytosis of the mucin-filled vesicles. We then examined the behavior of SNARE and interacting Munc18 proteins by confocal microscopy.

RESULTS: One and 6 μ mol/L carbachol evoked a dose-dependent dilation of Brunner's gland acini lumen, which correlated to the massive exocytosis of mucin. Munc18c and its cognate SNARE proteins Syntaxin-4 and SNAP-23 were localized to the apical plasma membrane, and upon cholinergic stimulation, Munc18c was displaced into the cytosol leaving Syntaxin-4 and SNAP-23 intact.

CONCLUSION: Physiologic cholinergic stimulation induces Munc18c displacement from the Brunner's gland acinar apical plasma membrane, which enables apical membrane Syntaxin-4 and SNAP-23 to form a SNARE complex with mucin-filled vesicle SNARE proteins to affect exocytosis.

INTRODUCTION

The Brunner's glands in the duodenum contribute to the discharge of mucin^[1,2] and also the secretion of a number of other products, including immunoglobulin, lysozyme, epidermal growth factor, and trefoil peptides, which collectively contribute to mucosal protection^[2-5]. Brunner's glands also secrete bicarbonate that contributes to the neutralization of the massive amount of acid coming from the stomach^[2], and duodenal alkalization is critical for pancreatic enzyme survival. The Brunner's gland is, therefore, a most versatile exocrine microorgan, but despite its importance in gastrointestinal health, relatively little is known about its secretory biology and regulation, in part because they exist as very small micro-organs buried within the duodenal mucosal epithelium.

To better understand Brunner's physiology, we previously reported the development of an *in vitro* model that permitted the examination of Brunner's gland secretion by video microscopy, which recorded real time changes in diameter of the dilating lumen of Brunner's gland acini that corresponded to the extent of mucin exocytotic emptying^[6,7]. Using this model, we demonstrated cholinergic stimulation of compound exocytosis of mucin into the ductal lumen, which was confirmed by electron microscopy and histology^[6]. We then went on to examine vagal neural cholinergic innervation^[6], and its coupling to potassium channel current, which regulated the acinar cell membrane excitability leading to secretion^[7].

Almost nothing is known about the molecular mechanisms regulating exocytosis *per se* in Brunner's gland acini. In contrast, there has been much insight into

molecular mechanism of exocytosis in the pancreatic acinar cell^[8,9]. It is very likely that similar exocytotic molecules in the pancreatic acinar cell would be conserved in Brunner's gland acini to mediate exocytosis of mucin. This led us to begin to explore for such exocytotic molecules, including SNARE (soluble NSF attachment protein receptor) proteins and associated Munc18 proteins, which regulate SNARE complex assembly^[10]. Munc18c binds Syntaxin-4 on the basolateral plasma membrane of the pancreatic acinar cell^[11]. Upon supramaximal cholinergic (or CCK) stimulation, Munc18c becomes phosphorylated causing its displacement from Syntaxin-4 into the cytosol, which activates Syntaxin-4 to bind SNAP-23, rendering the basolateral plasma membrane receptive to exocytosis by zymogen granules^[11]. We recently demonstrated this to be a contributing mechanism to supramaximal secretagogue-induced pancreatitis as well as alcoholic pancreatitis^[11-15].

In this work, we also found Munc18c, Syntaxin-4 and SNAP-23 to be present in Brunner's gland acini. Unlike pancreatic acini^[11-15], these exocytotic molecules are concentrated on the apical plasma membrane. Upon physiologic cholinergic stimulation, Munc18c behavior mimicked that of pancreatic acini^[11-15], becoming displaced from the apical membrane into the cytosol, which correlated to massive exocytosis of mucin into the dilating Brunner's gland acinar lumen.

MATERIALS AND METHODS

Antibodies and reagents

Antibodies used include those generated against Munc18c (a gift from Y Tamori, Kobe University, Japan), Syntaxin-4 (a gift from J Pessin, Stony Brook University, NY, USA), SNAP-23 (generated by us), and Mucin 5AC-clone 45M1 from Lab Vision (Fremont, CA, USA). Fluorochrome-conjugated secondary antibodies were from Jackson ImmunoResearch Laboratories (West Grove, PA, USA). All reagents were from Sigma Chemical Co. (St. Louis, MO, USA).

Brunner's gland preparation and stimulation by carbachol

In vitro submucosal preparations containing Brunner's glands were dissected from the duodenum of guinea pigs (150-200 g) of either sex, as previously described^[1,6,7]. Briefly, animals were anesthetized with isoflurane and killed by decapitation. The duodenum was opened along the mesenteric border and pinned flat with the mucosa side up in Sylgaard-lined petri dishes. The mucosa was dissected off and the underlying submucosa containing sheets of Brunner's glands dissected free from the circular muscle. Submucosal preparations were cut about 1 cm² and stored at room temperature (maximum time of 2 h) in physiological Krebs solution containing (in mmol/L): 126 NaCl, 2.5 NaH₂PO₄, 1.2 MgCl₂, 2.5 CaCl₂, 5 KCl, 25 NaHCO₃ and 11 glucose, equilibrated with 95% O₂-5% CO₂. Experiments were approved by the Queen's University and University of Toronto Animal Care Committees and met the guidelines of the Canadian Council of Animal Care.

In all studies, preparations were initially pinned in small

organ baths (1 mL), and superfused with Krebs's solution at 37°C for a 10 min equilibration period. They were then superfused for 3 min with Krebs's solution (control) or carbachol (1 µmol/L or 6 µmol/L). Tissues were then fixed by one of two means (see below) and coded to enable measurements to be performed in a blinded fashion.

Examination of exocytosis by transmission electron microscopy

Following superfusion with carbachol or control solution (Krebs's), preparations were fixed in 2% glutaraldehyde (pH 7.0) for 2 h and washed in sodium phosphate buffer. The fixed tissue was sectioned into segments about 2 mm² and immersed in 1% osmium tetroxide for 1 h. Tissue blocks were embedded in Eponar aldite. Brunner's glands were identified in semi-thin sections (0.5-1.5 µmol/L), which were cut perpendicular to the surface of the submucosal preparation and stained with toluidine blue. Plastic blocks were then trimmed to areas of about 0.5 mm² and ultrathin sections were cut and mounted on copper grids. These were stained with uranyl acetate and lead citrate and sections were viewed using a Zeiss EM 10 CR transmission electron microscope.

Confocal immunofluorescence microscopy

This was performed as similarly described in our previous reports^[11-15]. Frozen tissues were treated with Tissue-Tek OCT mounting compound from Tedpella (Redding, CA, USA), and then cut at 5 µm-thin sections. Frozen tissue sections were thaw-mounted onto VistaVision HistoBond adhesive slides from VWR (Mississauga, ON, Canada), fixed with 4% paraformaldehyde (1 h), rinsed in PBS and then treated with 100 mmol/L glycine (10 min). The tissues were then permeabilized with 0.1% Triton-X (in PBS), blocked with 5% albumin (overnight, 4°C) and then incubated with the primary antibody (1:50, 5 h). After extensive washing, fluorescently labeled secondary antibodies (1:1000) were added for 1 h, followed by washing, and treatment with Fluorescence Mounting Medium (DAKO Diagnostics, Mississauga, ON). The tissue samples were examined by a laser scanning confocal imaging system (Zeiss LSM510), equipped with LSM software version 5.00 (Carl Zeiss, Oberkochen, Germany).

Measurement of Brunner's gland dilation

Concurrent to obtaining the immunofluorescence images, DIC (differential interference contrast) images from frozen tissue sections were taken using the DIC optics of the confocal microscope. The apical lumens of the acini were measured using Image J software (NIH) by two independent observers in a blinded fashion. Significant differences between the two independent observers were not found and, therefore, the data were averaged for each measurement.

Statistical analysis

Slides were coded to avoid observer bias and measurements were performed in a blinded manner by two independent observers and their results averaged. All data are presented as mean ± SE. Statistical analysis was done

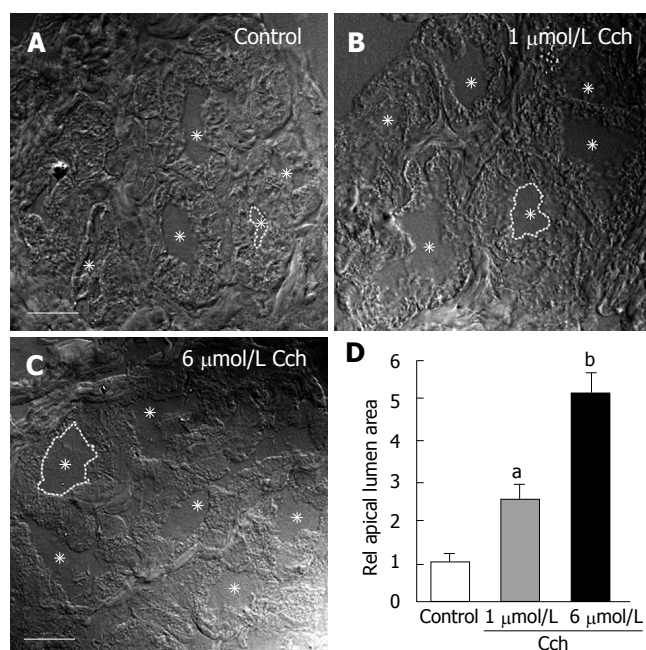


Figure 1 Carbachol-evoked dilatation of Brunner's gland acinar lumens. **A-C:** Representative differential interference contrast (DIC) images of control (**A**), 1 μmol/L Carbachol (**B**), and 6 μmol/L Carbachol (**C**) showing a dose-dependent dilatation of the acinar lumen. In each panel, the lumens are indicated by asterisks and one of the lumens is highlighted with a broken line. **D:** Statistical analysis of apical lumen area relative to control. For each condition 50 glands from 3 different sets of animals were measured (*n* = 150). Only those glands where the complete acinus was contained in the section were studied. Scale bar = 10 μm. ^a*P* < 0.05, ^b*P* < 0.01.

by Student's *t* test. Significance was assumed at a *P* value of less than 0.05.

RESULTS

Carbachol evokes a dose-dependent dilatation of Brunner's gland acinar lumens

Brunner's gland acinar cells contain mucin granules in the apical cytoplasm and previous studies have shown that stimulation-evoked dilation of Brunner's gland apical lumen is associated with the deposition of mucin in this space^[1]. In the current study, carbachol (1 μmol/L and 6 μmol/L) evoked a dose-dependent dilation of the lumen (Figure 1 A-C). Compared to controls (1.0 ± 0.2), carbachol 1 μmol/L caused 2.5-fold increase in luminal surface area (2.5 ± 0.5, *P* < 0.05) and 6 μmol/L caused a 5-fold increase (5.1 ± 0.7, *P* < 0.001; Figure 1D).

Carbachol evokes a dose-dependent mucin granule exocytosis into Brunner's gland acinar lumens

Transmission E.M. was employed to observe the effects of carbachol stimulation on the location and size of the mucin granules (Figure 2). In unstimulated preparations (Figure 2A), membrane-bound, electron-lucent vesicles were packed into the apical cytoplasm. Carbachol stimulation (1 μmol/L and 6 μmol/L in Figure 2B and 2C, respectively) caused fusion of the mucin vesicles deeper in the cytoplasm (indicated by asterisks, shown clearly in image blowups in Biii and Ciii), and migration and fusion of the vesicles with the apical membrane. Apical exocytosis included compound

exocytosis (indicated by asterisks, shown clearly in image blowups in Bii and Cii) which enabled very efficient extrusion of the mucin cargo into the lumen resulting in complete collapse of the vesicles and progressive luminal dilatation, which was massive with the 6 μmol/L carbachol stimulation. These findings, together with the measurements of acinar lumen, directly corroborate that carbachol activated compound exocytosis in these tissues.

Carbachol displaces Munc18c from the apical plasma membrane of Brunner's gland acinar cells

The distinct exocytotic events of Brunner's gland acini observed in Figures 1 and 2 are reminiscent of those in pancreatic acini^[8,9], and which we and others had reported to involve distinct SNARE and Munc18 proteins^[11-24]. We, therefore, probed the Brunner's gland acini with antibodies against Syntaxin (-2, -3 and -4), SNAP-23, VAMP (-2 and -8) and Munc18 (b and c), with the expectation that these exocytotic proteins would be differentially distributed in a manner reported in the rat pancreatic acinar cells^[8,9,11-24]. We also probed for mucin to verify its retention when unstimulated and its absence indicating exocytosis after stimulation. Importantly, these studies were conducted on the same tissues where carbachol-evoked dilation of the acinar lumen was demonstrated (see above), providing parallel functional evidence of exocytosis.

Of all these exocytotic protein antibodies, only Syntaxin-4, SNAP-23 and Munc18c antibodies showed consistent positive signals in the confocal immunofluorescence studies (Figures 3-5). Of note, all of these antibodies were raised against rat sequences and, therefore, the lack of detectable signals suggest that the antibodies may not recognize the guinea pig sequences or that those protein levels in guinea pig acini were not sufficiently abundant. In unstimulated acini, Syntaxin-4, SNAP-23 and Munc18c were abundant on the apical plasma membrane (Figure 3). This is surprisingly different from what had been demonstrated in pancreatic acini wherein these proteins were most abundant along the basolateral plasma membrane, and in fact, undetectable in the apical plasma membrane^[11-17]. Mucin lodged just beneath these exocytotic proteins, indicating their location in vesicles docked onto the apical pole of the acini. SNAP-23 signal seems to be also present in intracellular structures and the basal plasma membrane.

Upon 1 μmol/L (Figure 4) and 6 μmol/L carbachol (Figure 5) stimulation, Munc18c staining of the apical plasma membrane was progressively reduced. With 1 μmol/L carbachol stimulation (Figure 4), there was much cytosolic staining of Munc18c, but which was no longer present with 6 μmol/L carbachol (Figure 5). The latter is likely due to greater cytosolic proteolysis of Munc18c, which we had previously observed in rat pancreatic acini with supramaximal cholinergic stimulation^[11-14]. Our acini preparation would not permit us to obtain a large pure population of Brunner's gland acini required to perform biochemical analysis (Western blots) to detect Munc18c proteolytic products, or for the presence of the other exocytotic SNARE and Munc18b proteins. In contrast to Munc18c, Syntaxin-4 and SNAP-23 signals on the

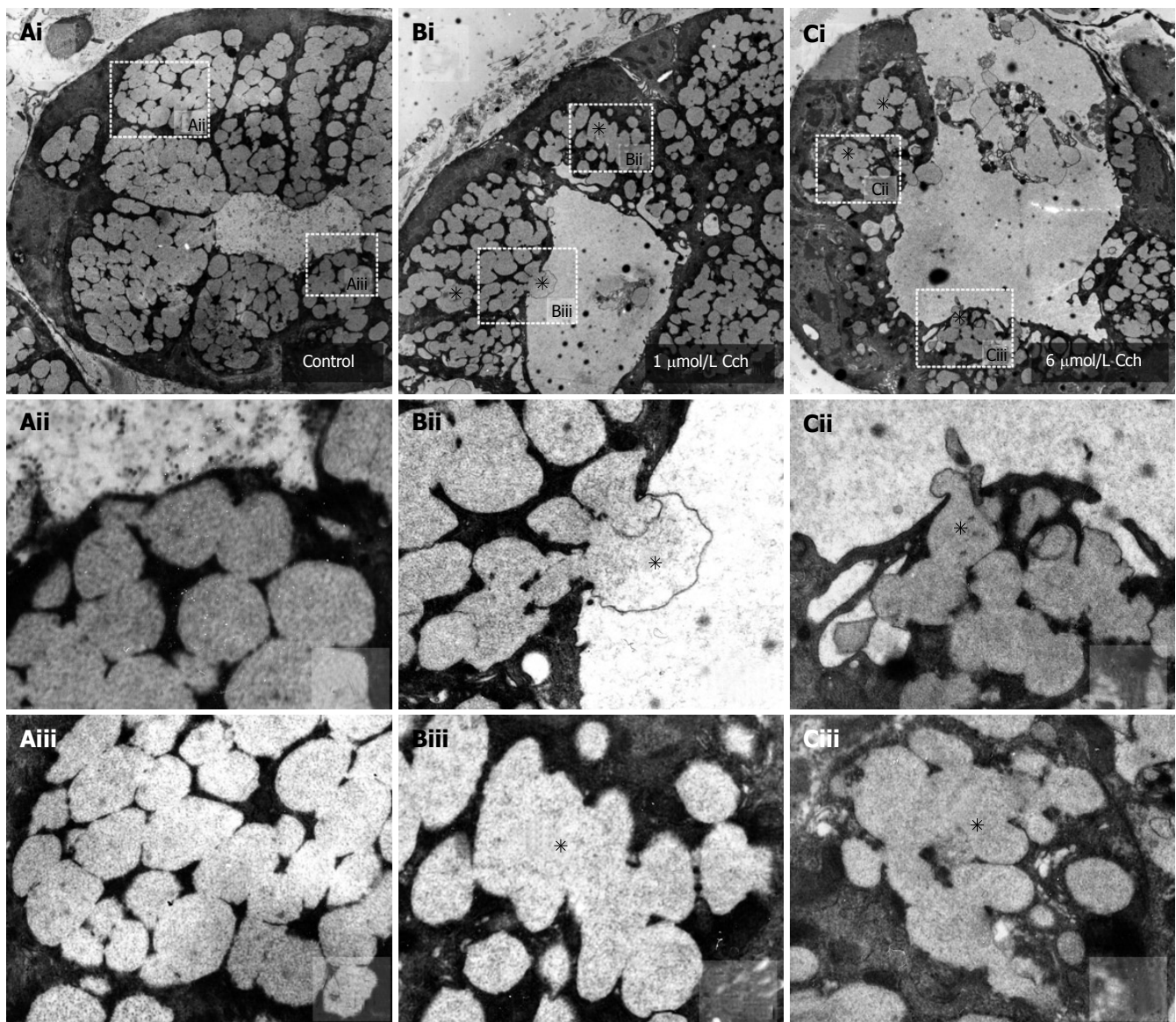


Figure 2 Carbachol-evoked mucin granule exocytosis into Brunner's gland acinar lumens. Representative transmission electron micrographs of Brunner's glands in unstimulated control (A), 1 $\mu\text{mol/L}$ (B) and 6 $\mu\text{mol/L}$ carbachol (C) stimulated tissue. **A:** Unstimulated Brunner's acinus ($\times 6250$). Aii and Aiii are blowups of indicated regions in Ai, showing small separate vesicles; **B:** One $\mu\text{mol/L}$ carbachol-stimulated Brunner's acinus showing a larger apical lumen with reduced number of electron-lucent mucin vesicles within the apical poles, with some vesicles undergoing vesicle-vesicle fusions (indicated by asterisk, $\times 6250$). Bii and Biii are blowups of indicated regions in Bi, showing compound exocytosis and vesicle-vesicle fusion (indicated by asterisks), respectively; **C:** Six $\mu\text{mol/L}$ carbachol stimulated Brunner's acinus. There is much less remaining vesicles some of which are undergoing vesicle-vesicle fusion (indicated by asterisk, $\times 5000$). Cii and Ciii are blowups of indicated regions in Ci, showing compound exocytosis and vesicle-vesicle fusion (indicated by asterisks), respectively.

apical plasma membrane after carbachol stimulation were as strong as unstimulated acini. As we had previously suggested in pancreatic acini, Munc18c displacement from the plasma membrane would enable Syntaxin-4 binding to SNAP-23 rendering the membrane receptive to exocytosis^[11-15]. Indeed, the mucin staining of the apical poles of the acini was very much reduced after 1 $\mu\text{mol/L}$ carbachol stimulation (Figure 4) and completely absent after 6 $\mu\text{mol/L}$ carbachol stimulation (Figure 5). This reduction or disappearance of acini mucin would correlate to the E.M. results (Figure 3) of the loss of electron-lucent mucin vesicles. The absence of mucin staining in the lumen, which would have been an indication of exocytosed mucin, is likely due to dilution of mucin within the lumen as to render the mucin signal below detection.

DISCUSSION

Using frozen sections of carbachol-stimulated submucosal preparation of Brunner's gland from guinea pig duodenum, we demonstrated the dilatation of acini lumen as an index of activation of secretion. These results are very similar to our previous reports using video-microscopy real-time recording of the real time changes of dilatation of acini lumen to track secretion^[1,6,7]. In those reports, we showed the range of cholinergic stimulation was between 0.1 $\mu\text{mol/L}$ to 10 $\mu\text{mol/L}$, with EC₅₀ of 2 $\mu\text{mol/L}$. Here, we used carbachol concentrations on the upstroke of the dose-response curve, that is 1 and 6 $\mu\text{mol/L}$, to demonstrate mild and near maximal dilatation of the ductal lumen. At 1 $\mu\text{mol/L}$ carbachol stimulation, E.M. performed on the same preparation

Control

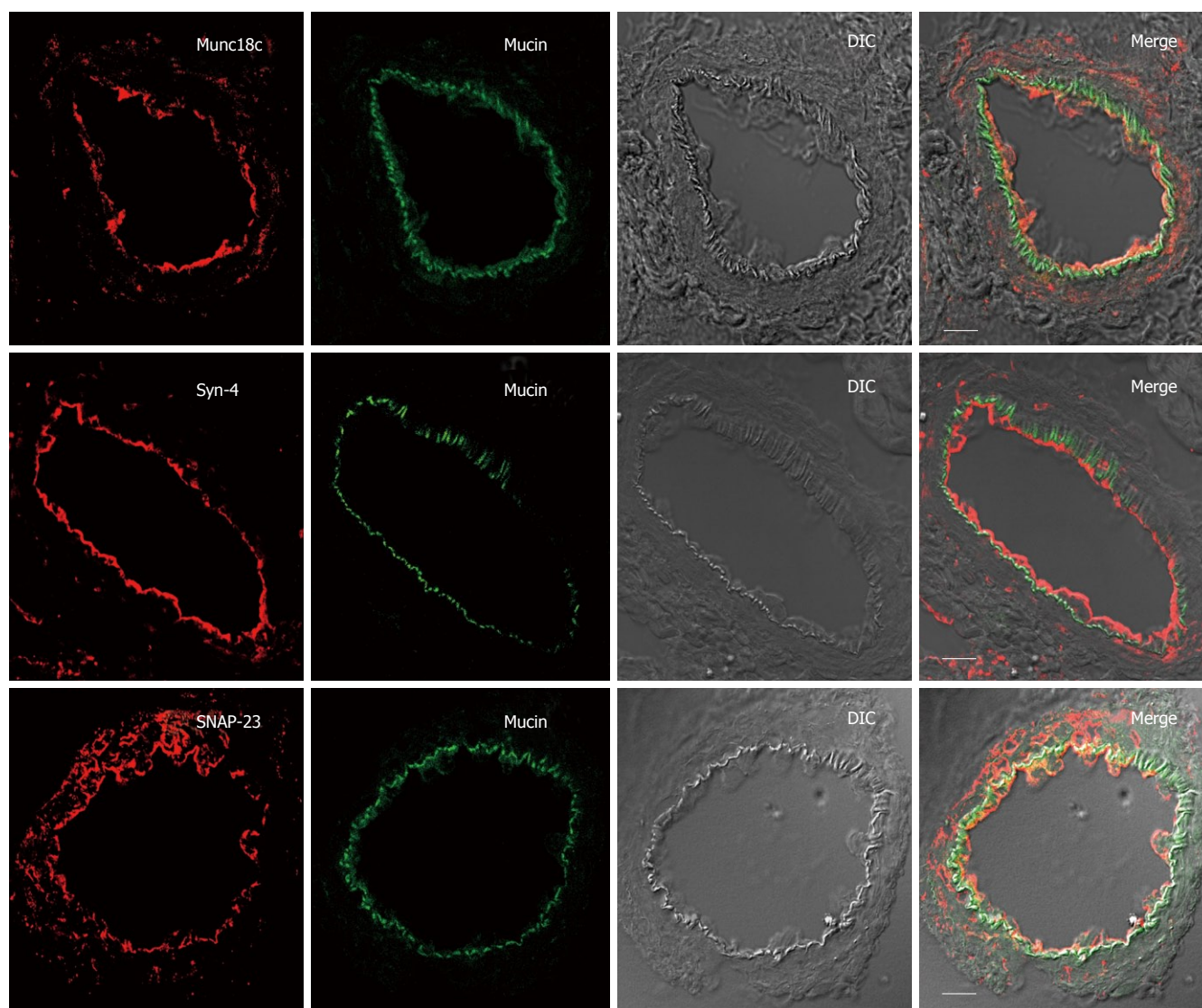


Figure 3 Apical SNARE localization in unstimulated Brunner's gland acini. Confocal images of unstimulated Brunner's gland acini labeled with anti-mucin antibody (in green) and double labeled with anti-Munc18c, Syntaxin-4 or SNAP-23 antibodies (in red), and an overlay (merge) of the two with the DIC, which is also separately shown. Note the apical plasma membrane staining of Munc18c (and SNAP-23 and Syntaxin-4) and mucin accumulation beneath it. Scale bars correspond to 10 μ m.

demonstrated some reduction of electron-lucent mucin containing vesicles and a mildly dilated ductal lumen. Here, many of the vesicles have undergone homotypic fusion and these prefused vesicles would surface to the apical plasma membrane to undergo compound exocytosis. These events were even more efficient at 6 μ mol/L carbachol stimulation, where we noted near total collapse and emptying of these mucin vesicles, leaving fewer apical vesicles. Here, the ductal lumen was massively dilated by the exocytosed mucin, the latter confirmed on our earlier report by PAS staining where upon stimulation the PAS positive material is found between acinar cells rather than within the apical cytoplasm as observed in unstimulated preparations^[1]. Due to anatomical reasons the best species to isolate Brunner's gland acini is the guinea pig as both the quantity and quality of the acini obtained are superior to that rendered by rat or mouse. This is also an excellent model to examine both hormonal regulation and neural innervation (which is intact in the preparation)^[1,6,7]; however, this preparation has some

disadvantages. We were not able to perform biochemical studies that require large homogeneous populations of acini, which was not possible to obtain with this preparation. This limitation was a problem in this study as all of antibodies used were against rat and mouse proteins, most of which were not appropriate for immunofluorescence studies of guinea pig acini, but would have been at least slightly positive on western blotting of purified membrane fractions of acini of rat and mouse.

It appears that the molecular machinery for exocytosis is conserved in Brunner's gland acini to mediate exocytosis. This machinery includes distinct combinations of syntaxins, SNAP-25 and VAMP proteins on cognate membrane compartments that would assemble into SNARE complex inducing fusion of the membranes^[11-14]. Munc18 proteins bind Syntaxins to prevent their interactions with the other SNARE proteins^[10]. Agonist stimulation of cells induces PKC mediated phosphorylation of Munc18 proteins, which activates the

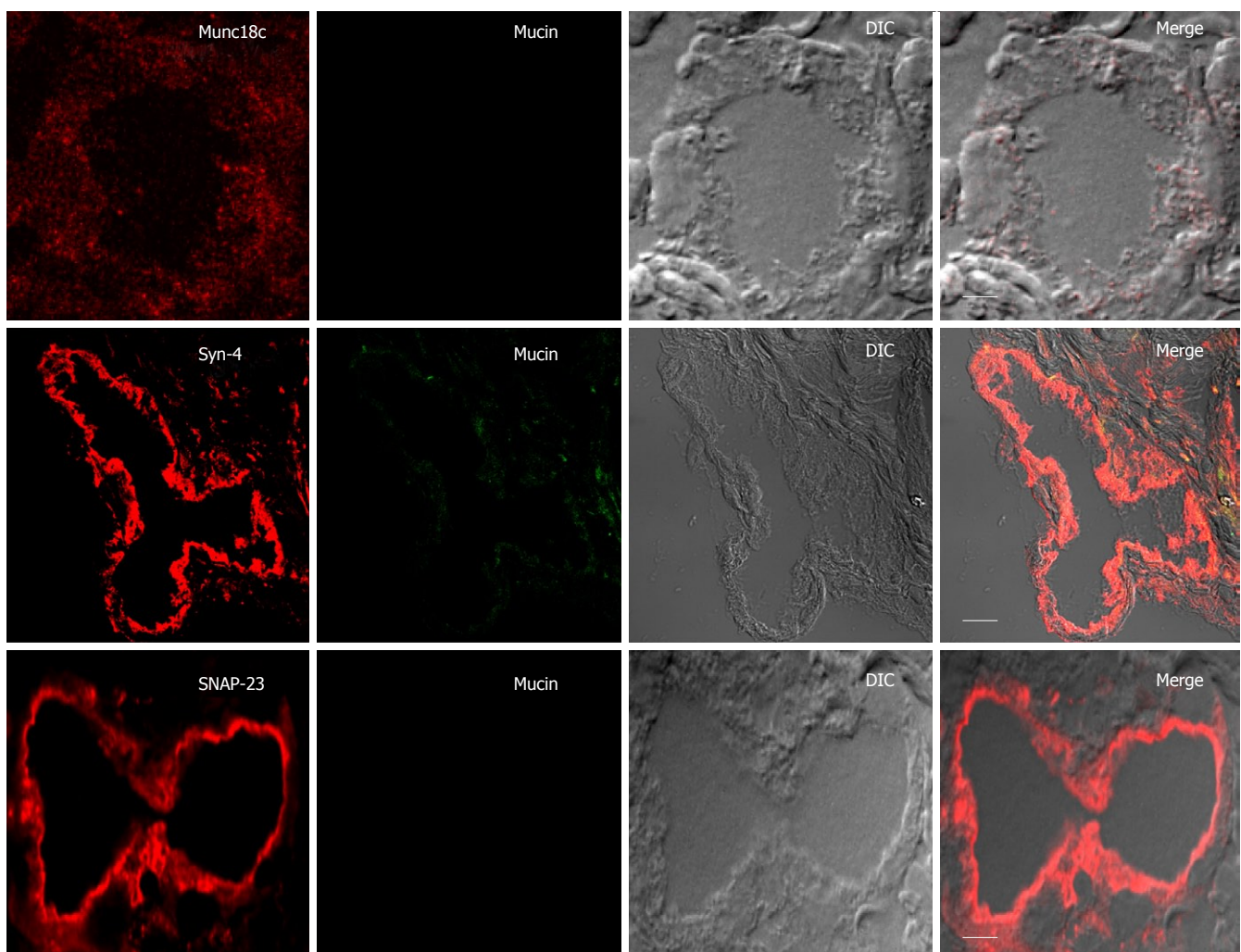
Cch (1 $\mu\text{mol/L}$)

Figure 4 SNARE localization after low carbachol (1 $\mu\text{mol/L}$) stimulation of Brunner's gland acini. Confocal images of 1 $\mu\text{mol/L}$ carbachol-stimulated Brunner's gland acini labeled with anti-mucin antibody (in green) and double labeled with anti-Munc18c, Syntaxin-4 or SNAP-23 antibodies (in red), and an overlay (merge) of the two with the DIC, which is also separately shown. Note the loss of apical plasma membrane staining of Munc18c with some staining in the cytosol, and much reduced mucin staining. Scale bars correspond to 10 μm .

Syntaxins into open conformations capable of binding SNAP-25 and VAMPs^[10]. We and others have previously demonstrated the distinct localization of homologs of these Munc18 (b and c) and SNARE proteins in rat pancreatic acini^[11-24]. Specifically, Syntaxin-2 is on the pancreatic acinar apical plasma membrane, Syntaxin-3 on the zymogen granules and Syntaxin-4 on the basolateral membrane. Munc18b was on the apical membrane and Munc18c on the basolateral membrane. We had shown that supramaximal cholinergic (or cholecystokinin) stimulation redirected apical exocytosis to the basolateral plasma membrane, a process requiring Munc18c displacement from Syntaxin-4^[11-15]. We postulated that Munc18c displacement activates Syntaxin-4 to form the exocytotic Syntaxin-4/SNAP-23/VAMP SNARE complex^[11-15]. In this work, we find that this molecular exocytotic machinery is also operational in Brunner's gland acini. However, it was surprising to see that this exocytotic machinery (Munc18c, Syntaxin-4 and SNAP-23) was localized to the apical plasma membrane of Brunner's

gland acini to mediate apical exocytosis. Of note, this exocytotic machinery also mediated apical exocytosis in rat parotid acinar cells^[25-27]. In contrast, rabbit lacrimal acini seem to follow a similar distribution for these exocytotic proteins as those described in rat pancreatic acini^[28]. In fact, like pancreatic acini^[11-14], apical exocytosis in rabbit lacrimal acini could also be redirected to the basolateral membrane surface by prolactin^[29]. The distinct localization of these exocytotic proteins within the family of exocrine tissues is of fundamental importance. Conventional thinking indicates that the localization of these proteins is conferred by signal sequences within these proteins^[30,31], which does not seem to be the case for the family of exocrine tissues. Additional cues likely from the membrane compartments themselves or from cytosolic chaperoning proteins that may be different between exocrine glands also seem to be required in determining the compartmental specific targeting of these exocytotic proteins. This notion is supported by our very recent report of a novel protein called Cab45b that is required to chaperone Munc18b to

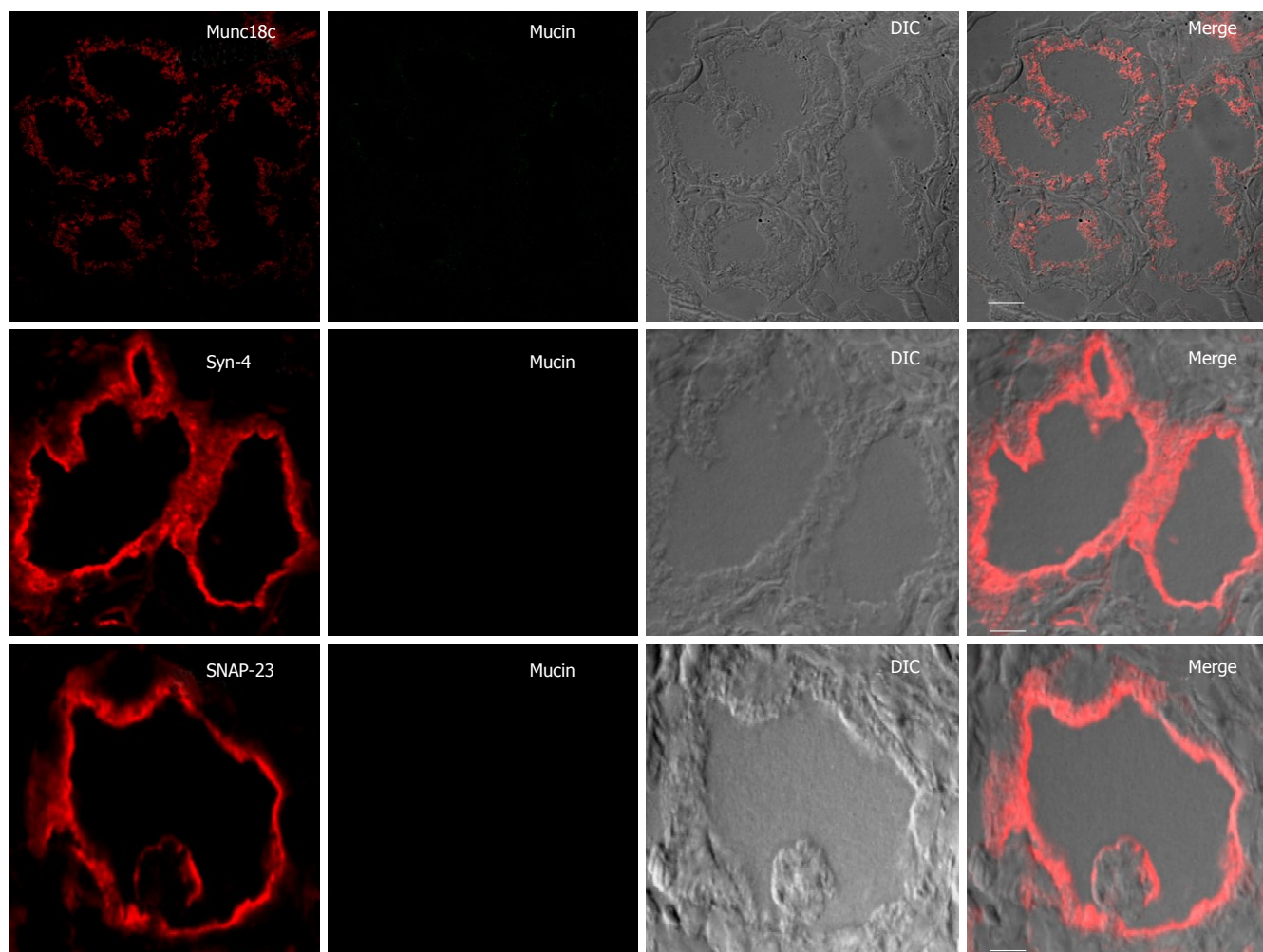
Cch (6 μ mol/L)

Figure 5 SNARE localization after high carbachol (6 μ mol/L) stimulation of Brunner's gland acini. Confocal images of 6 μ mol/L carbachol-stimulated Brunner's gland acini labeled with anti-mucin antibody (in green) and double labeled with anti-Munc18c, Syntaxin-4 or SNAP-23 antibodies (in red), and an overlay (Merge) of the two with the DIC, which is also separately shown. Note the further reduced apical plasma membrane staining of Munc18c (vs Figure 4) and the absence of mucin staining in the cells. DIC images exhibit dilated lumens. Scale bars correspond to 10 μ m.

its targeted compartments in pancreatic acinar cells^[19].

The behavior of activated Munc18c seems to be cell-context specific. In exocrine tissues (Brunner's gland, pancreatic and parotid acini), agonist stimulation resulted in Munc18c displacement from the plasma membrane into the cytosol as a prerequisite for Syntaxin-4 activation^[11-15,26]. This is, however, not the case for GLUT4 exocytosis in adipose tissues and skeletal muscles, which also use this set of exocytotic proteins^[32]. More recent reports suggest that Munc18c facilitates and actually stabilizes SNARE complex assembly^[33,34], a process that induces Syntaxin-4 into its open conformation^[35]. Although this process is associated with reduced Munc18c affinity to Syntaxin-4, the actual displacement of Munc18c from Syntaxin-4 is not required^[33,34]. Perhaps in acinar tissues, changes in Munc18c conformation after phosphorylation may render the 65 kDa Munc18c protein susceptible to proteolytic degradation^[11,14], and such cytosolic proteases may not be present or activated in adipose tissues and muscles. This possibility is supported by our reports in rat pancreatic acini demonstrating cytosolic Munc18c to be mostly a degraded 35 kDa product^[11-14].

While apical exocytosis in Brunner's gland acini is mediated by Munc18c/Syntaxin-4/SNAP-23, vesicle-vesicle fusions should be mediated by another exocytic machinery, likely involving Syntaxin-2 or -3 (and Munc18b) as is the case in pancreatic acini^[8,9,16,21]. As for vesicle VAMPs, this likely includes VAMP-2 and/or VAMP-8^[18,22,24,36]. Unfortunately, immunofluorescence studies we had carried out with a battery of antibodies directed against rat sequences (Munc18b, Syntaxin-2 and -3, VAMP-2 and -8) could not detect these proteins in the guinea pig Brunner's gland acini. Whether other secretory products (immunoglobulin, lysozyme, growth factors, trefoil peptides) of Brunner's gland acini^[3-5] are contained in the same mucin vesicles or distinct vesicle populations involving distinct sets of exocytotic proteins is particularly intriguing and would require further investigation.

ACKNOWLEDGMENTS

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COMMENTS

Background

Brunner's glands are small group of exocrine cells clustered together in small islands located in the duodenum where most peptic ulcers occur. These glands secrete bicarbonate and mucus (packaged in small vesicles) to protect the surrounding mucosa from injury that could be caused by the massive acid coming from the stomach.

Research frontiers

Exocytosis requires of membranes fusion. The SNARE Hypothesis implies minimal machinery for membrane fusion, including a cognate set of vesicle and target SNAREs on opposing membranes. Additionally, this hypothesis proposes that these SNARE proteins are prevented from spontaneous assembly by clamping proteins, here represented by Munc18c.

Innovations and breakthroughs

Brunner's glands have been extremely difficult to study until recently when we developed a duodenal submucosal preparation from guinea pigs. Using this preparation, we have now started to examine the molecules responsible for stimulated fusion of the mucus-containing vesicles with the plasma membrane, called exocytosis.

Applications

Here, we have determined the involvement of a group of intimately-interacting molecules (Munc18c, Syntaxin 4, SNAP-23) in the exocytosis of mucus. This set of molecules is very similar to that which mediates similar exocytotic processes in other exocrine glands in the body, including pancreatic (secreting digestive enzymes) and salivary glands (secreting digestive enzymes and mucus), which are important for food digestion and lubrication. This would indicate that these molecules are conserved in all exocrine glands to regulate secretions important for intestinal protection and food digestion, and which could be targeted for novel drugs to improve these important functions in the treatment of related disorders (peptic ulcer disease, pancreatic insufficiency, salivary gland disorders).

Peer review

The authors evaluated mechanisms to mediate exocytosis of Brunner's gland acini. Although many previous studies about exocytosis of pancreatic acinar cell were reported, as the authors mentioned, few were known about Brunner's gland acini. They demonstrated with sophisticated technique that Munc18c and SNARE proteins including Syntaxin-4 and SNAP-23 act like they do in the pancreatic acini, and speculated that the mechanisms are similar to those of pancreatic acini. Since a model of Brunner's gland in guinea pig duodenum is previously reported, the novelty of this report is to define the localization of Munc18c and SNARE proteins before and after cholinergic stimulus.

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Myelophil, a mixture of Astragali Radix and Salviae Radix extract, moderates toxic side effects of fluorouracil in mice

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evidence in support of clinical applications of Myelophil to minimize 5-FU-induced myelosuppression and improve general post-chemotherapy health.

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Abstract

AIM: To evaluate the efficacy of Myelophil, an extract containing Astragali Radix and Salviae Radix, for reducing complications induced by 5-fluorouracil (5-FU) in a gastrointestinal cancer model.

METHODS: We injected 5-FU into mice and then administered Myelophil to examine the ability of the drug to treat the side effects of 5-FU in mice. Peripheral blood counts, histological examinations, and colony-forming assays of bone marrow were conducted, followed by swimming tests and assessment of survival times.

RESULTS: Myelophil restored red and white blood cells and platelets in blood, and recovered cell density in bone marrow to levels comparable to those observed within the control group. In addition, Myelophil significantly increased colony-forming unit granulocyte-macrophage (CFU-GM) and CFU-erythroid (CFU-E) compared to the control group. We confirmed that interleukin-3 gene expression was upregulated by Myelophil in spleen cells. Myelophil administration also doubled the survival rate of mice that were severely myelosuppressed as a result of 5-FU injection at a lethal dose of 70%. Finally, the swimming performance of mice significantly improved as a result of Myelophil treatment.

CONCLUSION: These results provide experimental

INTRODUCTION

The occurrence of undesired effects that result from conventional chemotherapy or irradiation for cancer is inevitable. Nevertheless, reducing adverse effects is a critical issue for patients and doctors, given the importance of quality of life, as well as survival gains^[1-3]. Accordingly, mitigation of chemotherapy-induced side effects and the development of novel chemotherapeutic agents with fewer toxic effects have been major focuses of recent medical investigations^[4,5]. In particular, cancer-therapy-related fatigue, diarrhea or myelosuppression-related symptoms are closely associated with failure of the therapy itself. Therefore, many therapeutic developments, including herb-derived remedies, have focused on treating these side effects^[6-9].

Fluorouracil is one of the most commonly used drugs to treat gastrointestinal cancers, including those in the stomach, colon and liver, and it commonly causes fatigue, diarrhea and sometimes myelosuppression^[10,11]. Myelophil is a mixture of Astragali Radix and Salviae Radix extract representing Qi and blood, respectively, which support the liver and gastrointestinal system according to theories of Oriental medicine. Astragali Radix displays immunomodulating, hematopoietic, and anti-fibrotic properties^[12-14]. Salviae Radix exhibits antioxidant, antiatherosclerosis, and antiplatelet aggregation pharmaceutical effects^[15-17]. We have used this

drug to treat mainly gastrointestinal cancer patients with post-therapeutic complications such as leukopenia, anemia or severe fatigue since 2002.

Here, we evaluated the therapeutic efficacy of an extract mixture that contained *Astragali Radix* and *Salviae Radix* for reducing complications from cancer chemotherapy, using a 5-fluorouracil (5-FU)-induced myelosuppression mouse model.

MATERIALS AND METHODS

Manufacturing and fingerprinting of Myelophil

Astragalus membranaceus (Leguminosae, VS No: AM-2006-02-Ra) and *Salvia miltiorrhiza* (Labiatae, VS No: SM-2006-01-Ra) were provided by Daejeon Oriental Medical College, Dunsan Oriental Hospital, of Daejeon University, identified by Professor SI Yim of Daejeon University and stored at our laboratory for future use. Samik Pharmaceutical Company (Seoul, Korea) manufactured a lyophilized aqueous extract of Myelophil (mixture of *Astragali Radix* and *Salviae Radix*; 1:1) according to over-the-counter Korean monographs. A final product with 20.52% (w/w) yield was stored for future use (VS No: MP-2006-01-WE). 5-FU was purchased from Choongwae Pharma Corporation (Seoul, Korea). Other chemicals were obtained from Sigma (St. Louis, MO, USA).

For the fingerprinting of Myelophil, the water extract of *Astragali radix* and *Salviae radix*, and their standard components, formononetin (Sigma) and rosmarinic acid (Carl Roth, Karlsruhe, Germany) were prepared for the high performance thin layer chromatography (HPTLC) system (CAMAG, Muttenz, Switzerland). They were dissolved in HPLC-grade methanol and applied to pre-washed HPTLC plate silica gel 60 F₂₅₄ (Merck, Darmstadt, Germany) with an automated applicator (Linomat IV; CAMAG). The samples were then separated and the migrated components were visualized prior to or after derivatization under UV radiation at 366 nm or white light using Reprostar 3 with a digital camera (CAMAG, Figure 1).

Induction of myelosuppression: Hematological and histopathological analysis

To examine the therapeutic effects of Myelophil on 5-FU-induced myelosuppression, 60 6-wk-old male ICR mice (Koatech, Gyeonggi-do, Korea) were divided into three groups (20 control, and 20 with low- and 20 with high-concentration Myelophil treatment). Another five mice were sacrificed to record physiological standards for hematological parameters at time 0. All three groups were injected intraperitoneally with 0.3 g/kg 5-FU on d 0. Beginning on d 2, Myelophil (0.05 g/kg or 0.1 g/kg) or distilled water (control group) was administered orally once daily for 10 consecutive days. Five mice per group were serially sacrificed on d 0, 4, 7, 10 and 13, and complete blood counts were analyzed using a blood cell counter (HEMAVET; CDC Technologies, CT, USA). In addition, all left-side femoral bones from the mice on d 7 were prepared for general histopathological evaluation, including fixation, decalcification, sectioning (4 μ m thickness), as well as hematoxylin and eosin (HE) staining.

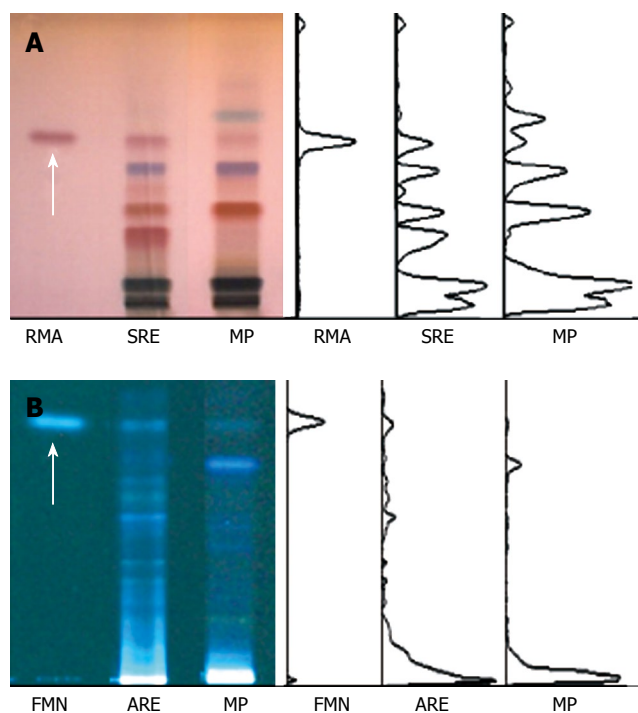


Figure 1 Fingerprinting of Myelophil. **A:** Rosmarinic acid (RMA, 2 μ g), *Salviae Radix* water extract (SRE, 200 μ g) and Myelophil (MP, 400 μ g) were separated using chloroform/ethyl acetate/benzene/formic acid/methanol (15:10:10:1), and then visualized by white light after derivatization with p-anisaldehyde sulfuric acid; **B:** Formononetin (FMN, 0.1 μ g), *Astragali Radix* water extract (ARE, 0.8 mg) and Myelophil (MP, 1.6 mg) were separated using dichloromethane/methanol/water (45:10:1), and then visualized under UV at 366 nm.

Isolation of bone marrow cells and colony-forming assay

To directly examine the effects of Myelophil on bone marrow stem cells, C57BL/6 mice (three for each of the control, and low- and high-concentration Myelophil groups) were injected intraperitoneally with 5-FU (0.2 g/kg). Mice were given Myelophil (0.05 g/kg or 0.1 g/kg) or distilled water (for naive and control groups) for 5 consecutive days beginning 2 d after 5-FU injection. Bone marrow cells were isolated from femurs, and nucleated cells were counted using a blood cell counter (HEMAVET; CDC Technologies). After thoroughly mixing the nucleated cells (400 μ L 2×10^5) with 4 mL MethoCult methylcellulose-based medium (Stem Cell Technologies, Seattle, WA, USA), media (1 mL per dish in triplicate) were cultured in a 5% CO₂ incubator for 7 d. According to the morphological characteristics, the number of colonies assessed by CFU-GM or CFU-E was counted under an inverted microscope.

Interleukin-3 (IL-3) gene expression analysis using real-time PCR

Splenocytes isolated from BALB/c male mice were seeded in a six-well culture plate (2.8×10^7 cells per well), and treated with or without Myelophil (0.001, 0.01 or 0.1 g/L) for 18 h. After purification of total RNA using an RNA mini kit (Qiagen, Valencia, CA, USA) and cDNA synthesis, quantitative real-time PCR was performed using SYBR Green Supermix reagent (Bio-Rad, CA, USA) according to the manufacturer's protocol. The primer sequences (forward and reverse, respectively) were

Table 1 Hematological effect of Myelophil on 5-FU-induced myelosuppression

Cells/groups		d 0	d 4	d 7	d 10	d 13
WBC (10^3 cells/ μ L)	Control	5.5 \pm 1.2	2.3 \pm 0.3	0.7 \pm 0.2	2.0 \pm 1.0	3.7 \pm 1.5
	MP 50	5.5 \pm 1.2	2.3 \pm 0.9	1.2 \pm 0.4 ^a	2.5 \pm 0.9	6.4 \pm 1.6 ^a
	MP100	5.5 \pm 1.2	2.4 \pm 0.3	1.6 \pm 0.4 ^b	2.9 \pm 0.6	4.2 \pm 1.0 ^a
RBC (10^6 cells/ μ L)	Control	7.1 \pm 0.8	7.3 \pm 0.6	5.3 \pm 0.2	6.3 \pm 1.2	7.0 \pm 1.0
	MP 50	7.1 \pm 0.8	6.3 \pm 0.6 ^a	5.9 \pm 0.4 ^b	6.9 \pm 0.7	6.9 \pm 1.2
	MP 100	7.1 \pm 0.8	6.6 \pm 0.5	6.6 \pm 0.4 ^b	6.7 \pm 0.5	7.2 \pm 1.0
Platelets (10^5 cells/ μ L)	Control	13.8 \pm 0.8	8.5 \pm 1.3	5.3 \pm 1.5	7.3 \pm 1.7	12.0 \pm 5.3
	MP 50	13.8 \pm 0.8	8.8 \pm 1.3	8.2 \pm 1.4 ^a	9.1 \pm 3.2	17.7 \pm 6.6
	MP 100	13.8 \pm 0.8	9.8 \pm 1.8	9.0 \pm 2.2 ^a	10.9 \pm 4.5	16.2 \pm 4.9

From 2 d after 5-FU injection, Myelophil (0.05 g/kg or 0.1 g/kg) or distilled water (for the control group) was given to mice for 10 d. On day 0, 4, 7, 10 and 13, complete blood counts were analyzed using a blood cell counter. Data are expressed as mean \pm SD ($n = 5$). ^a $P < 0.05$, ^b $P < 0.01$ vs control group. MP: Myelophil.

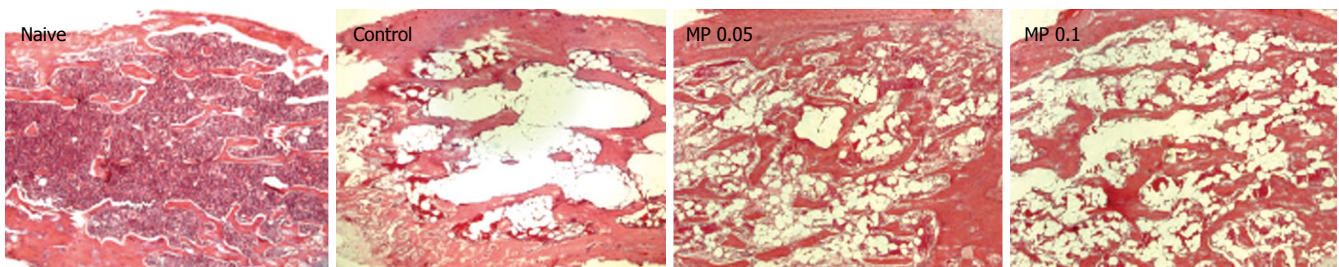


Figure 2 Histopathological analysis of bone marrow. After 7 d 5-FU injection (0.3 g/kg, intraperitoneally) and Myelophil treatment for 5 d, the femoral bone was dissected and fixed in 10% neutral-buffered formalin, followed by decalcification, embedding, and micro-sectioning (4 μ m). Histopathological examination was performed under a microscope ($\times 200$) after HE staining.

as follows: β -actin, GTGGGGCGCCCCAGGCACCA and CTCCTTAATGTCACGCACGATTTC; IL-3, TACATCTGCGAATGACTCTGC and GGCTGAGGTGGTCTAGAGGT.

Monitoring survival rates with or without swimming-forced stress

To examine whether Myelophil affected the survival time of mice with severe or moderate myelosuppression induced by 5-FU, we conducted two tests. First, severe myelosuppression was induced in 30 male ICR mice (10 in each of the control, and low- and high-concentration Myelophil groups) by 5-FU treatment (0.5 g/kg, intraperitoneally). Two days later, Myelophil (0.05 or 0.1 g/kg) or distilled water (induced group) was administered orally once daily for 10 consecutive days. The number of surviving mice was monitored for the next 20 d.

For the second test, moderate myelosuppression was induced in 30 male ICR mice (10 in each of the control, and low- and high-concentration Myelophil groups) by 5-FU treatment (0.3 g/kg, intraperitoneally). Beginning 2 d following 5-FU injection, Myelophil (0.05 or 0.1 g/kg) or distilled water (for the naïve and control groups) was given orally once daily for five consecutive days. On the final day, all mice were forced to swim in a pool with 22°C water for 30 min. Mice were monitored for survival time and swimming performance.

Statistical analysis

The results were expressed as mean \pm SD. Statistical

analysis of the data was conducted using Student's *t* test with significance levels of $P < 0.05$.

RESULTS

Hematological parameters

First, we examined changes in hematological parameters (leukocyte, erythrocyte and platelet counts) in 5-FU-induced (0.3 g/kg) myelosuppressed mice every 3 d. As shown in Table 1, peripheral blood white blood cell, platelet, and red blood cell levels drastically decreased, with the lowest numbers recorded on d 7. However, the observed pancytopenia was ameliorated by Myelophil administration, and the number of leukocytes rapidly recovered compared to that in untreated control mice (0.05 g/kg Myelophil, $P = 0.0387$; 0.1 g/kg Myelophil, $P = 0.0014$).

Histological examination of bone marrow

We examined the cellular density of femoral bone marrow from d 7 mice. Similar to the peripheral blood counts, 5-FU injection radically reduced the cellular component in bone marrow by vacuolation, and this was moderately improved by Myelophil treatment (Figure 2).

CFUs in bone marrow

To investigate how Myelophil affected the hematopoietic stem cells, leukocyte or erythrocyte-lineage colonies were determined using colony forming assay. Myelophil treatment significantly increased the colony numbers of both leukocyte (0.05 g/kg Myelophil, $P = 0.0087$; 0.1 g/kg Myelophil, $P = 0.0029$) and erythrocyte lineages (0.05 g/kg Myelophil,

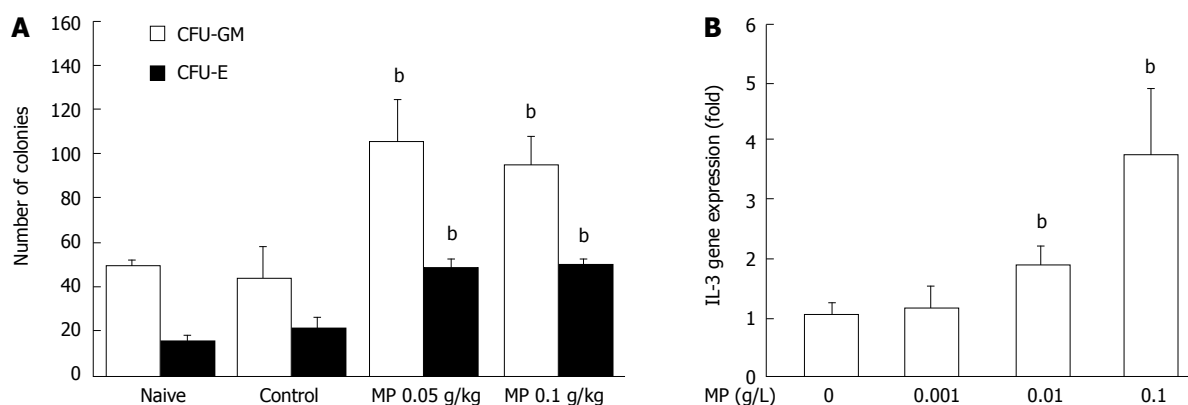


Figure 3 Colony-forming assay and IL-3 gene expression. **A:** After 7 d 5-FU injection (0.2 g/kg, intraperitoneally) and Myelophil treatment for 5 d, purified bone marrow cells were cultured for 7 d for colony counts of CFU-GM and CFU-E; **B:** Spleen cells were treated with Myelophil for 18 h, and then IL-3 gene expression was analyzed using real-time-PCR. Data are expressed as mean \pm SD ($n = 3$). ^b $P < 0.01$ vs control group.

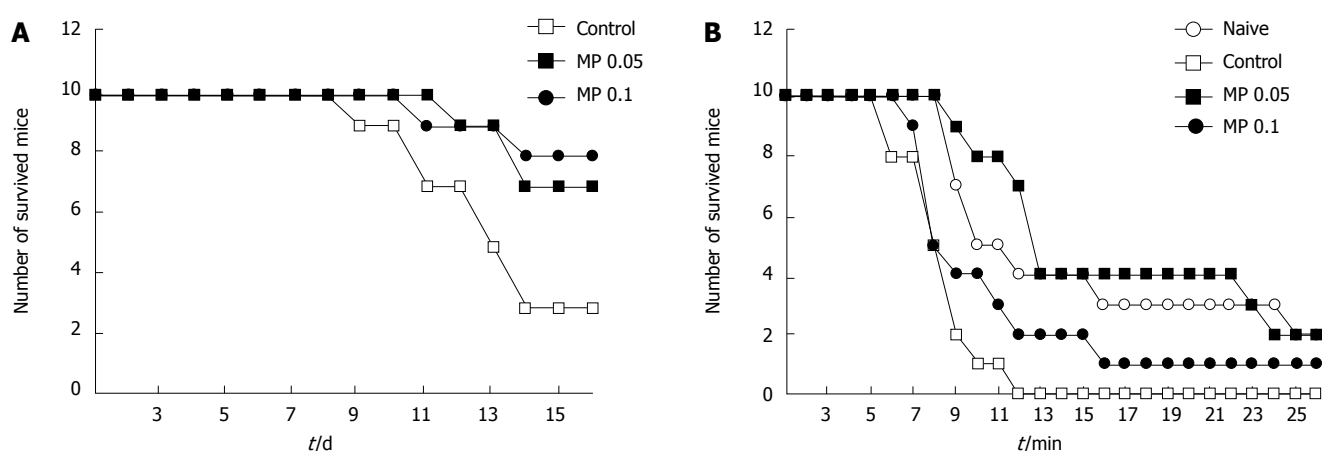


Figure 4 Survival of myelosuppressed mice. **A:** Beginning 2 d after 5-FU injection (0.5 g/kg, intraperitoneally), Myelophil (0.05 or 0.1 g/kg) or distilled water (control) was administered for 10 d. The number of surviving mice was monitored for 20 experimental days; **B:** Beginning 2 d after 5-FU injection (0.3 g/kg, intraperitoneally), Myelophil (0.05 or 0.1 g/kg) or distilled water (for naïve and control groups) was administered orally for 5 d. All mice were then forced to swim in a pool to monitor their survival time for 30 min.

$P = 0.0018$; 0.1 g/kg Myelophil, $P = 0.0021$), as shown in Figure 3A.

IL-3 gene expression in vitro

We examined changes in IL-3 gene expression in splenocytes following co-culturing with Myelophil using RT-PCR. IL-3 expression was increased in a dose-dependent manner. At a concentration of 0.1 g/L Myelophil, this gene was up-regulated four-fold (Figure 3B).

Survival rate of myelosuppressed mice

Following injection with 0.5 g/kg 5-FU (LD70, determined in our experiments), the survival rate of the control group was 30% by 14 d, whereas in the Myelophil-treated groups it was 70%-80% (Figure 4A). Myelophil treatment significantly protected mice from loss of body weight after 5-FU injection (data not shown). In addition, Myelophil treatment extended the survival time of mice that were forced to swim in a pool after injection with 0.3 g/kg of 5-FU (Figure 4B).

DISCUSSION

We used a 5-FU-induced myelosuppression mouse model

to evaluate the experimental efficacy of Myelophil, with relevance to the clinical application of reducing chemotherapy-induced side effects. 5-FU is one of the most commonly used anti-metabolic chemotherapeutic drugs for colon, stomach, liver, and head and neck cancer. It has also been applied in anti-myelosuppressive studies due to its observed toxicity toward bone marrow^[18-20]. We observed that a single injection of 0.5 g/kg 5-FU caused > 70% mortality within 20 d, whereas 0.3 g/kg 5-FU induced mild myelosuppression with no incidence of death.

Peripheral white blood cell, platelet and red blood cell levels drastically decreased in 5-FU-induced (0.3 g/kg) myelosuppressed mice. On d 7, these levels were the lowest and were in accordance with those of severe leukopenia, moderate thrombocytopenia and mild anemia. However, the observed pancytopenia was ameliorated by Myelophil administration, and the number of leukocytes rapidly recovered compared to that in untreated control mice. Next, we examined the cellular density of femoral bone marrow from d 7 mice. Similar to the peripheral blood results, 5-FU injection radically reduced the cellular component in bone marrow induced large vacuole formation, and this was moderately improved

by Myelophil treatment (Figure 2). These results suggest that Myelophil might be beneficial for the alleviation of chemotherapy-associated high susceptibility to pathogenic microorganisms, which is a major problem post-treatment^[21,22].

Consequently, we investigated how Myelophil affected the hematopoietic stem cells *via* differential examination of leukocyte- or erythrocyte-lineage colonies. The number and lineage of a colony was decided mainly by quantity and quality of stem cells in different groups. Our results showed Myelophil treatment significantly increased the colony numbers of both leukocyte and erythrocyte lineages (Figure 3A). Processes of hematopoiesis are under the control of various hematopoietic growth factors, such as IL-3, erythropoietin (EPO), thrombopoietin (TPO), granulocyte colony-stimulating factor (G-CSF), or granulocyte/macrophage CSF (GM-CSF)^[23]. These growth factors have lineage-specific hematopoietic functions and different cellular excretion sources^[24]. Specifically, IL-3 supports proliferation and differentiation of hematopoietic stem cells, as well as various cell lineages in hematopoiesis^[25], and is secreted mainly from natural killer T cells^[26]. Therefore, we examined changes in IL-3 gene expression in splenocytes following co-culturing with Myelophil. The result showed that IL-3 expression was increased four-fold (Figure 3B).

Given the above results, we observed how Myelophil could restore myelosuppression via IL-3 up-regulation. Generally, myelosuppression is linked strongly to other common side effects caused by conventional cancer therapy, such as fatigue or low energy, as well as low immunity.

Myelophil-treatment significantly protected mice from loss of body weight after 5-FU (0.5 g/kg) injection (data not shown). In addition, Myelophil treatment extended the survival times of mice that were forced to swim in a pool after injection of 0.3 g/kg 5-FU (Figure 4B).

5-FU-induced myelosuppression is the dose-limiting toxicity associated with substantial life-threatening risk and life span of cancer patients^[27,28]. Many herbal medicines are currently being investigated as good candidates for improving quality of life and reducing toxic side effects such as myelosuppression^[29-32]. One group has reported the efficacy of one of the two medicinal plant extracts in Myelophil on hematopoiesis induction in mice^[14]. We found that a mixture of Astragali Radix and Salviae Radix was more effective than a single herb administered alone in our model system (data not shown). We have prescribed Myelophil to treat post-therapeutic complications such as anemia, leukopenia or severe fatigue, mainly for gastrointestinal cancer patients, according to its oriental pharmaceutical theory since 2002.

Herein, we have provided experimental evidence relevant to clinical applications of Myelophil for minimizing cancer chemotherapy-induced side-effects, using a fluorouracil-induced myelosuppression mouse model.

COMMENTS

Background

Anticancer-therapy-induced side effects are closely associated with life span and quality of life in cancer patients, so reducing or preventing these has been a major

issue in cancer treatment. Astragali Radix and Salviae Radix extract, Myelophil, has been used to treat mainly gastrointestinal cancer patients with post-therapeutic complications such as leukopenia, anemia or severe fatigue since 2002. This study demonstrated the efficacy of Myelophil for moderating the toxic side effects of 5-FU.

Research frontiers

Fluorouracil is one of the most commonly used drugs to treat gastrointestinal cancers, including stomach, colon and liver, but it commonly causes fatigue, diarrhea and sometimes myelosuppression. Myelophil significantly ameliorated the toxic side effects of 5-FU, such as leukopenia, anemia and thrombocytopenia and improved survival rate in myelosuppressed mice.

Innovations and breakthroughs

Myelophil showed dramatic activity against the side effects of 5-FU, a very widely used drug. This study showed evidence that a herbal remedy can be a good candidate for improving quality of life in cancer patients.

Applications

Myelophil may be of benefit to cancer patients suffering from chemotherapy-induced side effects.

Peer review

This manuscript examines two herbal extract mixtures that reduce complications induced by the chemotherapeutic agent, 5-FU in a gastrointestinal cancer model. The bone marrow data, colony-forming assay, and interleukin gene expression all support the moderating effects of Myelophil on toxic side effects of 5-FU, and show the possibilities for clinical applications.

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Proliferation of L02 human hepatocytes in tolerized genetically immunocompetent rats

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Abstract

AIM: To investigate whether human hepatocytes could proliferate after transplantation to normal immunocompetent rats treated with 2-acetaminofluorene or Retrorsine and partial hepatectomy.

METHODS: L02 hepatocyte-tolerant Sprague-Dawley rats were injected with Retrorsine, 2-acetaminofluorene or normal saline. L02 hepatocytes were then transplanted *via* the spleen. Human albumin and its mRNA, specific proliferating cell nuclear antigen (PCNA), L02 hepatocyte dynamic distribution, number density and area density of PCNA-positive cells in the liver were determined.

RESULTS: All the examined indicators were not significantly different between the rats treated with 2-acetaminofluorene and normal saline, which was not the case with rats treated with Retrorsine. A dynamic distribution of L02 hepatocytes in the rat liver was detected from wk 1 to mo 6 after transplantation in the Retrorsine group and from wk 1 to 10 in the 2-acetaminofluorene group. Human albumin and its mRNA were detected from wk 2 to mo 6 in the Retrorsine group and from wk 1 to 8 in the 2-acetaminofluorene group. Specific human PCNA was detected in the rat liver from wk 2 to mo 6 in the Retrorsine group and from wk 2 to 6 in the 2-acetaminofluorene group. Human albumin and its mRNA contents as well as the number of PCNA positive cells reached a peak at wk 4.

CONCLUSION: L02 human hepatocytes could not proliferate significantly after transplantation to the normal, immunocompetent rats treated with 2-acetaminofluorene.

L02 human hepatocytes can survive for 10 wk after transplantation and express human albumin for 8 wk. L02 human hepatocytes can proliferate and express human albumin for 6 mo after transplantation to the rats treated with Retrorsine. The chimeric L02 human hepatocytes, which then underwent transplantation into tolerant rats, were normal in morphogenesis, biochemistry and function.

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Key words: Hepatocyte; Chimerism; Rat; Transplantation; Proliferation

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INTRODUCTION

An animal model of rats with chimeric human livers has been created with human hepatocytes being transplanted into rat livers. These rats can be used to identify hepatitis virus with the problem of species-specificity being resolved^[1]. With immunodeficiency^[2] or induced immunotolerance rats^[3], an animal model of rats with chimeric human liver infected with Hepatitis B virus can be established. An animal model of tolerant rats with chimeric human liver can be applied in pathogenesis, antiviral therapy and the development of vaccines^[4]. However, human hepatocytes transplanted into rats can survive only a limited time. The lifetime of transplanted hepatocytes must be prolonged to establish a persistent animal model. With an exogenous growth stimulus, such as partial hepatectomy, the recipient hepatocytes can not only regenerate but the transplanted hepatocytes can also proliferate^[5]. If restraining the regeneration of recipient hepatocytes with 2-acetaminofluorene (2-AAF), Retrorsine(Rts), dipin, furan, or 3'-diethoxycarbonyl-1,4-dihydrocollidine (DDC)^[6-8], followed by an exogenous growth stimulus such as carbon tetrachloride(CCl₄) or partial hepatectomy (PH)^[9-11], the proliferation of transplanted hepatocytes can be improved significantly. We investigate whether human hepatocytes could repopulate

after transplantation to rats with a normal immune system treated with 2-acetaminofluorene (2-AAF) or Retrorsine (Rts) and partial hepatectomy (PH).

L02 Human hepatocytes were injected into the peritoneal cavities of fetal Sprague-Dawley rats to induce immune tolerance to L02 human hepatocytes. The 2- or 3-wk rats were injected with 2-AAF or Rts. The L02 human hepatocytes stained with 1,1'-Diiodo-3,3',3''-tetramethylindocarbocyanine perchlorate (DiI) were transplanted into the spleen via intrasplenic injection. The survival and proliferation of human L02 hepatocytes in the rat liver were analyzed by an immunofluorescence method, reverse transcription-polymerase chain reaction (RT-PCR), immunohistochemical study, DiI fluorescence tracing and computer-aided image analysis.

MATERIALS AND METHODS

Materials

Nine Sprague-Dawley rats at reproductive age were purchased from the Third Military Medical University. Rats at gestational age 15-17 d and weighing 250-300 g were used. They were maintained on an alternating 12-h light/dark cycle with food and water available *ad libitum*. All studies were conducted under protocols approved by the Laboratory Animal Resource Center of the Third Military Medical University, China.

L02 hepatocytes, the isolated normal human hepatocytes, which have not been immortalized, were purchased from Shanghai Institute of Cell Biology of Chinese Academy of Science. L02 hepatocytes were digested with pancreatin, centrifuged twice at 1100 r/min for 3 min, and then resuspended. Hepatocytes were counted using a blood cell counting chamber and cell viability was assessed by trypan blue exclusion assay. Cells with viability above 80% were used in the study.

Methods

Induction of immunotolerance of fetal rats: Sprague-Dawley rats at gestational age 15-17 d were etherized, their abdomens were cut open along the linea alba abdominis, and their uterus was exposed. 50 μ L of human L02 hepatocytes suspension (1×10^8 cells) were slowly injected into the abdominal cavities of the 8-12 fetal rats in utero using a 1 mL-syringe, and then the abdominal walls of the pregnant rats were sutured layer by layer. Pregnant rats were fed till spontaneous delivery.

Drug solution preparation: The drug solution was prepared as described elsewhere^[12]. Retrorsine (Sigma Company) was added to distilled water at 10 mg/mL and titrated to pH 2.5 with 1 mol/L HCl. The solution was neutralized with 1 mol/L NaOH, and NaCl was added for a final concentration of 6 mg/mL retrorsine and 0.15 mol/L NaCl (pH 7). The working solution was used immediately after preparation. 2-acetaminofluorene was dissolved in M400 polyethylene glycol.

Animal grouping and drug injection: Pregnant rats were randomized to three groups, namely, the experimental (2AAF and Rts) group and the control group, and 30

fetal rats were assigned to each group. For the Rts group, immunotolerant rats were intraperitoneally injected with retrorsine (30 mg/kg/rat) 3 and 5 wk post birth. For the 2AAF group, immunotolerant rats were intraperitoneally injected with 2-acetaminofluorene (30 mg/kg/rat) 2 wk post birth, once every other day, total 4 injection. For the control group, immunotolerant rats were intraperitoneally injected with physiologic saline and the other procedures were the same as the experimental group. 7 wk post birth, rats of the Rts group and 3 wk post birth, rats of the 2AAF group and the control group were etherized and underwent 2/3 hepatectomy and transplantation of 100 μ L of DiI-labeled human L02 hepatocyte suspension ($> 10^{12}$ cells/L) through the spleen.

DiI fluorescence staining: The method was described previously^[13]. Five μ L of 1 mmol/L Vybrant DiI (Sigma Company) cell-labeling solution dissolved in 100% ethanol was added to 1 mL of culture medium. Cells were stained for 40 min at 37°C in a CO₂ incubator. Labeled cells were centrifuged three times by 1100 revolutions per minute (r/min) \times 3 min and washed with phosphate-buffered saline (PBS). They were then counted by trypan blue exclusion assay and suspended at 1.0×10^{12} /L with PBS. Cells were observed and photographed under the fluorescence microscope (Olympus Company) with a rhodamine filter.

L02 hepatocytes transplantation: 3 or 7-wk-old fetal rats of the experimental group were etherized. The abdomen was cut open along the linea alba abdominis and the spleen was exposed. 100 μ L of DiI-stained L02 hepatocyte suspension was slowly injected along the mesenteric edge into the splenic body and tail. The injection site was pressed gently to prevent hemorrhage or effusion.

Specimen collection: Specimens were collected one week after transplantation, and then at wk 2, 4, 6, 8 and mo 4 and 6. Specimens were collected from four rats at each collection point. Under anesthetization, rats underwent partial hepatectomy, and fresh liver tissue was subjected to frozen sectioning. Sections were observed under the fluorescence microscope with a rhodamine filter. Some tissue was fixated in 40 g/L neutral formalin, embedded in paraffin and sectioned. After surgery, rats were fed as usual.

DiI fluorescence tracing: L02 hepatocytes were stained with DiI and observed under the fluorescence microscope with a rhodamine filter before transplantation. Following L02 hepatocytes transplantation, fresh rat liver tissue was subjected to frozen sectioning and was also observed under the fluorescence microscope.

Detection of human albumin in liver tissue by immunofluorescence method: Fresh liver tissue was subjected to frozen sectioning with a thickness of 4-8 μ m. The sections were placed at room temperature for 30 min and fixated at 4°C in acetone for 10 min. Unspecific staining was blocked at $< 37^\circ\text{C}$ with 100 mL/L blocking serum for 20 min. The sections were then added to rat anti-

human albumin monoclonal antibody (Sigma Company, 1:400) and kept at 4°C overnight. They were subsequently added to fluorescence labeled a secondary antibody, i.e., Fluorescein isothiocyanate (FITC)-labeled goat anti-rat IgG (Beijing Zhongshan Company, 1:100), and incubated at 37°C in darkness for 45 min. The sections were mounted with 900 mL/L buffering glycerol and observed under a laser confocal microscope (Swiss Zeiss Company). For negative control, the primary antibody was replaced by PBS.

Immunohistochemical detection of human PCNA in the liver tissue: Streptavidin peroxidase conjunction (S-P) method was used. In brief, fresh liver tissue was fixated in 40 g/L neutral formalin, embedded in paraffin and sectioned, baked at 60°C for 1 to 2 h, and underwent deparaffinage. The sections were added with PBS containing 0.5 g/L Triton \times 100 and incubated at room temperature for 5 min. Then the sections were incubated with 3% hydrogen peroxide at 37°C for 20 min to block endogenous peroxidase. The sections were incubated with specific monoclonal antibody against human proliferating cell nuclear antigen (PCNA) (Chemicon Company, 1:200) or non-specific monoclonal antibody against PCNA (Beijing Dingguo Company, 1:200, against vertebrate PCNA) was added and incubated at 4°C overnight, then polymer boost and horseradish peroxidase-labeled goat anti-rat IgG multimer (Beijing Zhongshan Company) was added. The next steps included 3,3'-Diaminobenzidine (DAB) coloration, after-stain with hematoxylin, mounting with neutral resin, and observation under a light microscope. For negative control, the primary antibody was replaced by PBS.

Cell image analysis: The LeicaQWin image analysis system (Germany Leica Company) was used. Five areas were randomly selected in each section at the magnitude of 100 and the number density (cell/ μm^2) and area density ($\mu\text{m}^2/\mu\text{m}^3$) of PCNA-positive cells were calculated.

RT-PCR detection of human albumin mRNA in the rat liver tissue: This step was carried out according to the instructions for use of the Trizol RNA extraction kit. The two-step method using RT-PCR kit (BioDev Company) was adopted for reverse transcription and amplification. Primers were signed and synthesized by Takara Company (Dalian, China). Human albumin mRNA primers: forward primer Hs: 5'-TCGACAACGGCTCCGGCAT-3', reverse primer Ha: 5'-AAGGTGTGGTGCCAGATTTC-3', length of the amplified fragment: 241 bp; normal rat albumin mRNA primers: forward primer Rs: 5'-CGGT TTAGGGACTTAGGAGAACAGC-3', reverse primer Ra: 5'-ATAGTGTCCCAGAAAGCTGGTAGGG-3', length of the amplified fragment: 388 bp. Amplification was carried out by using the following cycle: initial denaturation at 94°C for 5 min, denaturation at 94°C for 50 s, reassociation at 55°C for 50 s, extension at 72°C for 1 min (32 cycles), and a final extension step at 72°C for 8 min. To exclude false positive results, negative control was set by amplification without AMV reverse transcriptase. The products were subjected to 1.5%

agarose gel electrophoresis, ethidium bromide staining and image acquisition using a gel scanner (America Bio-Rad Company).

Statistical analysis

Data were expressed as mean \pm SD. Paired *t*-tests were adopted to compare group differences using SPSS 10.0.

RESULTS

Anesthetic overdose caused one death in the experimental (2AAF) group and two deaths in the control group. The remaining rats survived.

DiI fluorescence tracing

DiI-stained cells gave off red fluorescence seen through a green filter. They were round or elliptical, and evenly stained, and their nuclei could not be differentiated from cytoplasm. At wk 1, 2, 4, 6 and 8 and mo 4 and 6, the distribution of L02 hepatocytes in the rat liver was dynamically observed under the fluorescence microscope in the Rts group. Transplanted L02 hepatocytes distributed in the rat liver parenchyma and weak fluorescence dispersed evenly from wk 1 to mo 4. At mo 6, the number of cells with fluorescence and fluorescence intensity began to decrease, and fluorescence distributed evenly in patches. There was no significant difference between the 2AAF and the control group. Transplanted L02 hepatocytes first distributed in mass in the portal area of the host rat, and then scattered to liver parenchyma. Compared to the Rts group, at the same time point, the number of cells with fluorescence was significantly reduced in the 2AAF or the control group. The number of cells with fluorescence decreased over time and the fluorescence intensity was also weakened over time. Cell fluorescence persisted till week 10 (Figure 1).

Detection of human albumin in the rat liver tissue by immunofluorescence method

FITC was used to label the secondary antibody, and the positive cell cytoplasm gave off green fluorescence under the laser confocal microscope through the blue filter, and there was a distinct boundary between positive cells and adjacent ones. In the Rts group, at wk 2, 4, 6 and 8 and mo 4 and 6, human albumin was detected, with most cells giving off green fluorescence at week 4. In the 2AAF and the control group, human albumin was detected at wk 2, 4, 6 and 8, but not in 4 rats throughout 10 wk. With time, there was more human albumin at the same time point in the Rts group than in the 2AAF and the control group. In the Rts group liver parenchyma exhibited mild to moderate destruction of hepatic cord, inflammatory cell filtration of the portal area and dispersed enlarged hepatocytes with enlarged nuclei. However, in the 2AAF and the control group these phenomena were not significant. Hepatic fibrosis or liver necrosis was present in neither group (Figure 2).

Immunohistochemical detection of human PCNA

Human PCNA-positive nuclei appeared brown after DAB coloration (Figure 3). The primary antibody was specifically

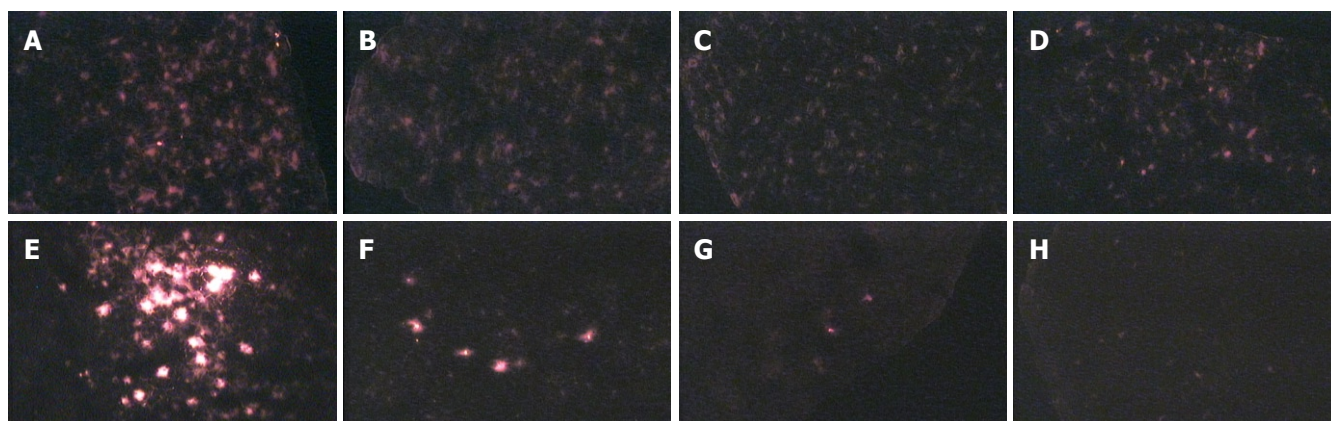


Figure 1 Fluorescence image of DiI-stained L02 hepatocytes ($\times 200$). **A-D**: The Rts group at wk 1, 4, 8 and mo 6 after transplantation; **E-H**: The 2AAF group at wk 1, 4, 8 and mo 6 after transplantation.

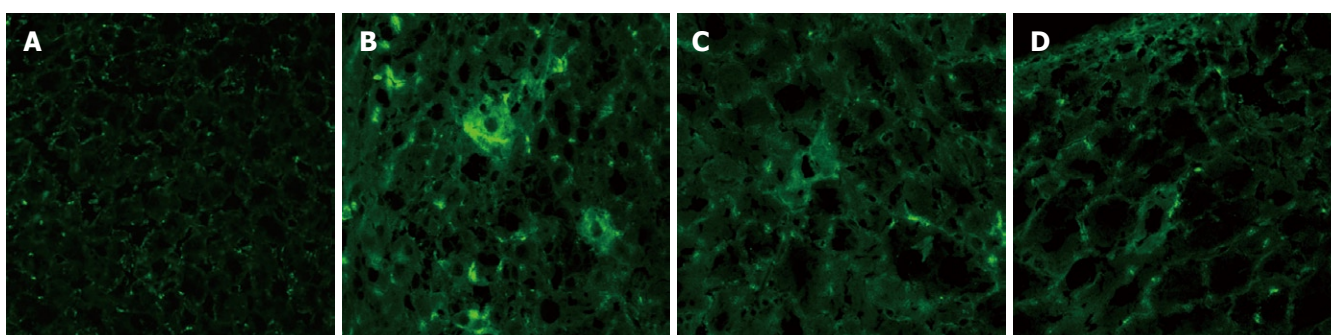


Figure 2 L02 hepatocytes giving off green fluorescence in the rat liver tissue with chimeric human hepatocytes at wk 4 after transplantation ($\times 400$). **A**: Normal rat liver tissue; **B**: The Rts group; **C**: The 2AAF group; **D**: The control.

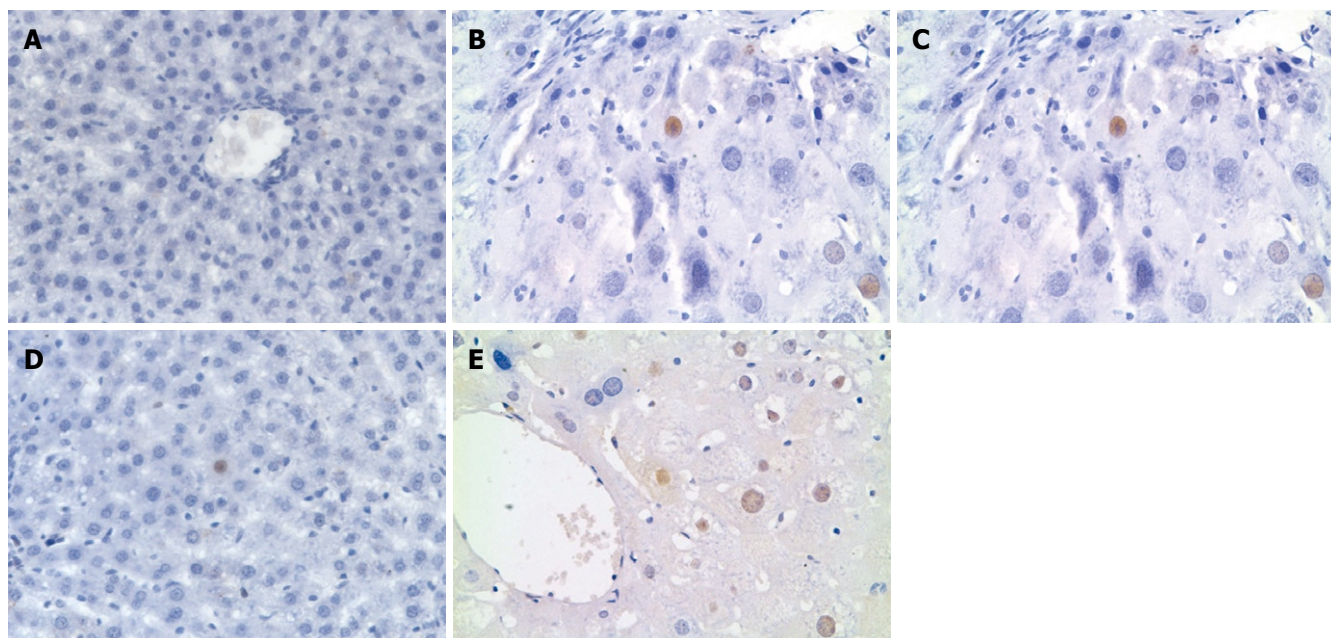


Figure 3 L02 hepatocytes with brown nuclei in normal rat liver tissue and rat liver tissue with chimeric human hepatocytes at wk 4 after transplantation ($\times 200$). **A**: Normal rat liver tissue; **B**: The Rts group; **C**: The 2AAF group; **D**: The control; **E**: Primary antibody not specifically against human PCNA.

against human PCNA, and there was no human PCNA detected in the rat liver tissue 1 wk after transplantation. Human PCNA was detected in the Rts group at wk 2, 4, 6 and

8, and mo 4 and 6, with the most detected at wk 4. Human PCNA then gradually decreased, however, still appeared at mo 6. In the 2AAF and the control group, human

Table 1 Number and area density of PCNA-positive cells at different time points (100 ×, mean ± SD, 10⁴ cells/μm²)

Index	Subgroup		1 wk	2 wk	4 wk	6 wk	8 wk	6 mo
Number density (10 ⁴ μm ² /μm ²)	Human PCNA	H-Rts		0.47 ± 0.08 ^a	1.33 ± 0.34 ^a	0.56 ± 0.38 ^a	0.08 ± 0.03	0.11 ± 0.07
		H-2AAF		0.14 ± 0.03	0.43 ± 0.04	0.26 ± 0.08		
		H-control		0.10 ± 0.07	0.46 ± 0.04	0.14 ± 0.04		
	Nonspecific PCNA	N-Rts	5.53 ± 1.12	5.68 ± 0.89	6.02 ± 0.87	6.78 ± 1.03	5.77 ± 0.68	5.95 ± 1.73
		N-2AAF	6.26 ± 1.07	5.79 ± 0.75	6.32 ± 0.84	7.58 ± 1.17	5.18 ± 1.03	
		N-Control	6.20 ± 0.85	5.77 ± 1.18	6.16 ± 1.02	5.46 ± 1.03	6.73 ± 0.58	6.28 ± 0.73
Area density (10 ⁴ μm ² /μm ²)	Human PCNA	H-Rts		2.14 ± 0.22 ^a	7.85 ± 1.46 ^a	3.54 ± 1.63 ^a	0.58 ± 0.72	1.24 ± 0.04
		H-2AAF		1.58 ± 0.27	2.36 ± 0.26	1.17 ± 0.22		
		H-Control		1.47 ± 0.04	2.94 ± 0.64	2.37 ± 0.48		
	Nonspecific PCNA	N-Rts	74.8 ± 1.06	69.3 ± 2.84	45.3 ± 3.38	77.3 ± 4.84	84.7 ± 2.67	73.7 ± 5.38
		N-2AAF	54.6 ± 2.56	49.9 ± 3.37	52.7 ± 4.46	47.5 ± 3.58	51.4 ± 3.27	
		N-Control	84.2 ± 6.84	67.5 ± 2.73	66.3 ± 4.48	74.2 ± 5.73	83.3 ± 4.83	66.3 ± 5.74

^a*P* < 0.05 vs Control.

PCNA was detected at wk 2, 4 and 6, with the most detected at wk 4. With a primary antibody not specifically against human PCNA, human PCNA was detected in the rat liver tissue 1 wk after transplantation. In the Rts group, a great amount of non-specific human PCNA was detected at wk 1, 2, 4, 6 and 8, and mo 4 and 6, with no decrease in the number of PCNA-positive nuclei. In the 2AAF and the control group, a great amount of non-specific human PCNA was detected at wk 1, 2, 4, 6 and 8, with no changes in the number of PCNA-positive nuclei.

Analysis of PCNA-positive cell images

Number density of PCNA-positive cells at various time points were calculated by computer-aided image analysis and were then subjected to statistical analysis. Using the primary antibody specifically against human PCNA, there were significant differences in the number or area density between the Rts and the control group. There were no significant differences between the 2AAF and the control group. While using the primary antibody not specifically against human PCNA, there were no significant differences between either groups (Table 1). The curve chart of number density of PCNA-positive cells is presented in Figure 4.

RT-PCR detection of human albumin mRNA

Human *albumin* mRNA was detected at wk 2, 4, 6 and 8, and mo 4 and 6 in the Rts group and at wk 2, 4, 6 and 8 in the 2AAF and the control group. Human *albumin* mRNA band (241 bp) and rat *albumin* mRNA band (388 bp) were detected by RT-PCR in the rat liver tissue using relevant human and rat *albumin* mRNA primers (Figure 5).

DISCUSSION

Rodent animal models especially play an important role in the development of hepatology. The hepatitis virus has species-specificity and, susceptible hosts are confined to higher grade primates, such as humans, macaques and chimpanzees^[14] for various reasons. With immunodeficient or induced immunotolerant rats, rat animal models with chimeric human liver infected with hepatitis virus can be established. During embryonic development, the immune system is not mature, and tolerance of exogenous antigens can occur due to "T lymphocyte clonal deletion"^[15].

Hence, animal models for graft tolerance may be established by induction of fetal rat immunotolerance against human hepatocytes by using human fetal hepatocytes and then transplantation of human hepatocytes into normal rat liver^[16]. An animal model of tolerant rats with chimeric human hepatocytes was established based on the normal immune system, and the chimeric cells in the rat liver were normal human hepatocytes, which are susceptible to many hepatitis-causing agents. Therefore, this rat liver model may be used for studying known hepatitis-causing agents such as HBV, HCV, and HEV virus as well as unknown hepatitis-causing agents.

With the use of labeling technique, transplanted hepatocytes can be easily differentiated from host hepatocytes under the fluorescence microscope or by flow cytometry, and the function, turnover, distribution and survival of transplanted hepatocytes can be monitored directly. We used the lipophilic fluorescent dye DiI to label the whole cell. DiI fluorescence attenuated slowly. It was reported that DiI-labeled neurons can give off fluorescence for more than 4 wk *in vitro* and maintain activity for more than 9 mo *in vivo*. Fluorescence did not attenuate significantly or diffuse to adjacent neurons^[17]. In this study, DiI fluorescence tracing and immunohistochemical study demonstrated that transplanted L02 hepatocytes first migrated to the distal portal area and liver sinuses in mass in host rat liver, then to liver parenchyma and were then scattered. Under the action of retrorsine, scattered L02 hepatocytes began to proliferate in clusters. In the Rts group, L02 hepatocytes scattered evenly with weak fluorescence during wk 1 after transplantation, suggesting that these cells migrated to the portal area and underwent cell division immediately after migrating to liver parenchyma, leading to weakened fluorescence. In the 2AAF and control groups, the fluorescence intensity was relatively high at the same time point, but the number of cells with fluorescence was significantly small compared to the experimental group, and both were gradually decreased, suggesting less cell divisions. The results demonstrated that retrorsine can significantly promote transplanted L02 hepatocytes to proliferate, but 2AAF cannot. After integrated into the parenchymal plates, transplanted hepatocytes exhibited albumin and enzyme expression and metabolism of biliary. It was observed that there was full reconstitution of gap junction

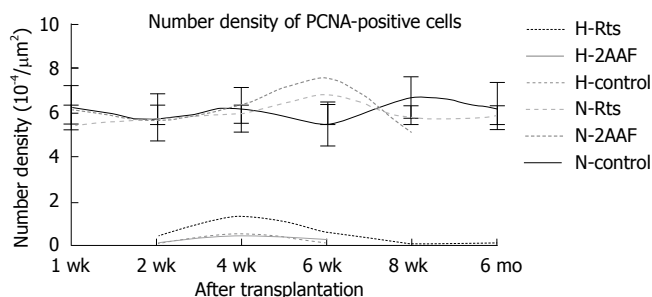


Figure 4 Curve chart of number density of PCNA-positive cells number density of PCNA-positive cells. Using the primary antibody specifically against human PCNA, there were significant differences in the number density between the Rts and the control group, there were no significant differences between the 2AAF and the control group, while using the primary antibody not specifically against human PCNA, there were no significant differences between either groups.

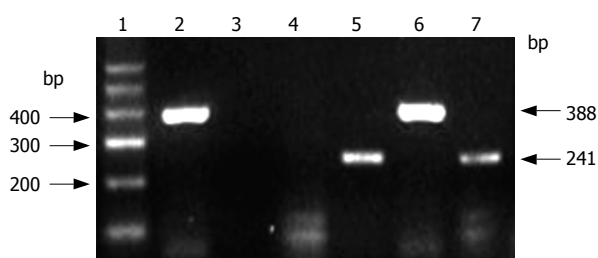


Figure 5 RT-PCR detection of human and rat albumin mRNA in liver tissue. 1: Standard molecular weight DNA marker; 2, 3: Normal rat liver tissue; 4, 5: Human liver tissue; 6, 7: Rats liver tissue with chimeric human hepatocytes at wk 2, 4, 6, 8, and mo 4, 6 in the Rts group and at wk 2, 4, 6, 8 in the 2AAF and the control group; 2, 4, 6: Rat *albumin* mRNA primers used in the RT-PCR reaction system, amplified 388 bp fragment of rat *albumin* mRNA presented in the rat liver tissue of 2 (normal rat liver tissue) and 6 (rats liver tissue with chimeric human hepatocytes), but not in 4 (human liver tissue); 3, 5, 7: Human *albumin* mRNA primers used in the RT-PCR reaction system, the amplified products did not present in 3 (normal rat liver tissue), but in 5 (human liver tissue) and 7 (rats liver tissue with chimeric human hepatocytes), amplified 241 bp human albumin mRNA presented in the rat liver tissue.

and biliaral struction between the transplanted and host hepatocytes 5 d after transplantation. This means that successful integration needs at least 5 d^[18]. In our study, the cells with fluorescence were detected 1 wk after transplantation but human albumin and mRNA were not detected, suggesting that transplanted L02 hepatocytes did not have the ability of human albumin exhibition and proliferation yet, though had been transferred and intergrated into the parenchymal plates. Cell fluorescence lasted for more than 6 mo in the Rts group and 8 wk in the 2AAF and control groups, confirming that DiI attenuates very slowly. In the 2AAF and control groups, disappearance of fluorescence was not due to attenuation but to limited proliferating capability of transplanted cells and decreases in cell viability.

In this study, by using immunofluorescence and RT-PCR, we detected human *albumin* and human *albumin* mRNA at wk 2, 4, 6 and 8, and mo 4 and 6 in the rat liver tissue with chimeric human hepatocytes in the Rts group and at wk 2, 4, 6 and 8 in the 2AAF and control groups. This further confirms the presence and biological function of human L02 cells in the chimeric rat liver. Immunological rejection normally happened 3 wk after transplanta-

tion^[19]. So we could conclude that the dissolution of transplanted cells was not due to immunological rejection with the host, but to limited proliferating ability of transplanted hepatocytes^[20]. The presence of human L02 hepatocytes persisted significantly longer in the Rts group than in the 2AAF and control groups. Hepatic fibrosis or necrosis was observed in neither group, suggesting that retrorsine promotes L02 hepatocyte proliferation in the rat liver tissue, and that hepatotoxicity of retrorsine does not affect the biochemical function of transplanted cells. In the Rts group, destruction of liver cord, remarkable inflammatory cell filtration and hepatocytes with enlarged nuclei in liver parenchyma were observed; however, these phenomena were not obvious in the 2AAF and control groups. These findings reflected the integration of transplanted hepatocytes and host cells as well as gradual proliferation and replacement of transplanted cells. The enlarged hepatocytes in rats exposed to retrorsine are interpreted to result from endoreduplication of host cells, which are able to duplicate DNA but cannot proceed through mitosis. Consistent with this interpretation, formation of enlarged cells is dependent on the presence of a stimulus for cell division. The hepatocytes of normal size and appearance could originate either from transplanted or host hepatocytes that were able to withstand the inhibitory effect of retrorsine or from progenitor cells that are unaffected by retrorsine and can differentiate into seemingly normal hepatocytes^[21]. Laconi *et al* found that since total weight of liver and DNA content did not exceed the normal range during the proliferation of transplanted cells, the transplanted cells not only simply coexist with host cells but replace them^[22]. Besides retrorsine, transplanted cells per se may also accelerate the replacement of host cells.

Retrorsine and 2/3 hepatectomy (Rts/PH) in combination are frequently used to stimulate proliferation of the transplanted hepatocytes. A two-thirds hepatectomy stimulates chimeric liver regeneration and increases the rate of host cell renewal. Retrorsine potently and persistently blocks the cell cycle of host hepatocytes, but does not affect the cell cycle of transplanted hepatocytes, resulting in stronger growth vigor of transplanted hepatocytes. However, the precise mechanism involved remains unclear. It was shown that compared to injection of retrorsine, injection of a great number of hepatocytes ($> 1 \times 10^8$ cells) into the spleen, repeated transplantation of hepatocytes or induction of liver toxicity before cell transplantation influenced hepatocyte proliferation mildly^[23]. Although the toxicity of retrorsine may lead to hepatocyte necrosis, it did not affect hepatocyte proliferation, thus shedding light on efficient proliferation of transplanted hepatocytes and the clinical application of retrorsine.

Retrorsine is a pyrrolidine alkaloid derived from Senecio plants, which may potently and persistently block the cell cycle of proliferating host hepatocytes, while not affecting the cell cycle of proliferating transplanted hepatocytes, thus enhancing the selective growth of transplanted hepatocytes. Despite a short half life period, retrorsine may suppress hepatocyte proliferation for weeks and even months^[24]. Retrorsine mainly blocks phases S and G2 of the liver cell cycle; that is, it blocks cell mitosis. Moreover, it may prevent hepatocytes from entering phase S, binding

to DNA, and blocking DNA synthesis^[25]. Other DNA-binding agents such as diethylnitrosamine cannot maintain the proliferation of transplanted hepatocytes, indicating the peculiarity of alkaloid^[22]. Laconi *et al* found that in the absence of exogenous growth stimuli, such as partial hepatectomy, retrorsine treatment may give rise to results similar to those of a combination of retrorsine treatment and partial hepatectomy, suggesting that besides maintenance of selective growth vigor of transplanted hepatocytes, there may be another mechanism of retrorsine action^[26]. Gordon *et al* investigated the mechanism by which retrorsine causes elimination of irreversibly damaged giant hepatocytes, finding that retrorsine can block liver cell mitosis and results in the accumulation of cells at late phase S and phase G2, and induces apoptosis and cell injury depending on the relative levels of the proapoptotic protein Bax and the anti-apoptotic protein Bcl-xl^[27].

2AAF is a hepatotoxic drug that blocks DNA synthesis and suppresses proliferation of host hepatocytes, finally activating the proliferation of oval cells^[28,29]. In our study, we investigated whether 2AAF can promote the proliferation of transplanting human hepatocytes in the tolerant rat liver.

The computer-aided image analysis system has been widely used to analyze immunohistochemical images and calculate the number, number density, area, and area density of transplanted hepatocytes, maximum cell diameter and other morphological parameters. In our study, the number density and area density of PCNA positive cells at various time points were calculated by computer-aided image analysis and were then subjected to statistical analysis. It was found that, using the primary antibody specifically against human PCNA, human PCNA was observed at wk 2, 4 and 6 both in the 2AAF and control group, showing that human L02 cells in proliferative phase survived in the rat livers.

Image analysis revealed that there were not significant differences in the number density and area density between both groups, suggested that 2AAF could not promote the proliferation of transplanted cells. In both groups, the highest number of proliferating L02 hepatocytes was observed at wk 4, showing the strongest proliferating capability at wk 4. The proliferating L02 hepatocytes disappeared at wk 8, but fluorescence tracing and immunohistochemical results showed that transplanted hepatocytes still survived and owned some functions at wk 8. Image analysis revealed that there were significant differences in the number density and area density between both groups, suggesting that retrorsine significantly promoted the proliferation of transplanted cells. Human PCNA was observed at wk 2, 4, 6 and 8, and mo 4 and 6 in the Rts group and at wk 2, 4 and 6 in the control group, and human PCNA persisted significantly longer in the Rts group than in the control group, suggesting that retrorsine can inhibit the proliferation of host hepatocytes and promote the activation of oval cells and the proliferation of transplanted hepatocytes. Zheng *et al* postulated that regeneration of severely injured liver following retrorsine treatment may first involve the proliferation and differentiation of transplanted adult hepatocytes, and then the proliferation and differentiation of mono- or bi-potent precursor cells^[30].

In the Rts group, the highest number of proliferating

L02 hepatocytes was observed at wk 4, with significantly more cells in the Rts group than in the control group, and following this the number gradually dropped. At mo 4 after transplantation, there was a small number of positive cells. This number was similar to that at mo 6, suggesting that the high-level differentiation and proliferation of L02 hepatocytes in the rat liver persisted till mo 4, and L02 hepatocytes still replicated at low levels during late stages. Zheng *et al* transplanted DPPIV + hepatocytes into DPPIV-rats subjected to retrorsine treatment and partial hepatectomy, and they found that DNA replication and cell division were the most frequent at mo 1 and then subsided, and that a platform of cell proliferation was reached at mo 3, but with DNA replication remaining at a low level^[30]. Our findings are largely in agreement with their results.

Using a primary antibody not specifically against human PCNA, there were no significant differences in the persisting time and number of proliferating L02 hepatocytes between the experimental (Rts or 2AAF) groups and the control group. Non-specific PCNA presented in large amounts after wk 1, demonstrated that the proliferation of rat hepatocytes was remarkable and persistent following partial hepatectomy. Seventy percent hepatectomy is frequently used to reproduce the liver regeneration models in rats. Transplanted hepatocytes accounted for a minority of the cell population in the chimeric liver and partial hepatectomy caused the proliferation of just a few L02 cells. Moreover, even if retrorsine enhanced the proliferation of transplanted cells, specific human PCNA-positive cells were substantially fewer than non-specific PCNA-positive cells at the same time point. Non-specific PCNA was observed at wk 1, while specific human PCNA was not. Retrorsine permitted DNA synthesis in the host hepatocytes and mainly blocked hepatocytes at late phase S and phase G2; that is, it blocked cell mitosis and inhibited the proliferation of host hepatocytes. Through this mechanism^[25], hepatocytes with enlarged nuclei emerged following retrorsine treatment, partially because of replication and synthesis of DNA in host hepatocytes and failure of these cells to undergo mitosis, which indicates that selective proliferation of transplanted hepatocytes in retrorsine treated animals is dependent, at least in part, on the persistent cell cycle block imposed by retrorsine on host cells. 2AAF blocked DNA synthesis and suppressed proliferation of host hepatocytes, and mainly promoted the proliferation of oval cells. But in our study, 2AAF did not promote the proliferation of transplanted L02 hepatocytes.

Retrorsine promoted the proliferation of L02 hepatocytes in rat liver with chimeric human hepatocytes and prolonged cell survival, and thus a stable, persistent animal model was achieved. The study sheds light on the usefulness of rat liver models with chimeric human hepatocytes; the animal model based on the normal immune system may open up a new avenue of study on the immune response and pathogenetic mechanism of viral infection, and antiviral therapy as well as the development of potent vaccines.

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COMMENTS

Background

An animal model of rats with chimeric human liver is that human hepatocytes are transplanted into rat liver. But human hepatocytes transplanted into rats just can survive a limited time. The live time of transplanted hepatocytes must be prolonged to establish a persistent animal model.

Research frontiers

Retrorsine, or other hepatotoxicity drugs and 2/3 hepatectomy in combination are frequently used to stimulate proliferation of the transplanted hepatocytes. 2/3 hepatectomy stimulates chimeric liver regeneration and increases the rate of host cell renewal. Retrorsine potently and persistently blocks the cell cycle of host hepatocytes, but does not affect the cell cycle of transplanted hepatocytes, resulting in stronger growth vigor of transplanted hepatocytes. 2AAF is a hepatotoxicity drug to block DNA synthesis and suppress proliferation of host hepatocytes, finally activated the proliferation of oval cells.

Innovations and breakthroughs

The animal model of tolerant rats with chimeric human hepatocytes we established was based on the normal immune system, and the chimeric cells in the rat liver were normal human hepatocytes. With the use of Dil labeling technique, transplanted hepatocytes can be easily differentiated from host hepatocytes under the fluorescence microscope, and the function, turnover, distribution and survival of transplanted hepatocytes can be monitored directly. The survival and proliferation of human L02 hepatocytes in the rat liver were analyzed by immunofluorescence method, reverse transcription-polymerase chain reaction (RT-PCR), immunohistochemical study, and computer-aided image analysis.

Applications

The chimeric cells in the rat liver were normal human hepatocytes, which are susceptible to many hepatitis-causing agents. Therefore, this rat liver model may be used for studying known hepatitis-causing agents such as HBV, HCV, and HEV virus and unknown hepatitis-causing agents. The animal model of tolerant rats with chimeric human liver can be applied in pathogenesis, antiviral therapy and development of vaccine.

Terminology

An animal model of rats with chimeric human liver is that human hepatocytes are transplanted into rat liver. With induced immunotolerance rats, it can establish animal model of rats with chimeric human liver infected with Hepatitis B virus, which can be applied in pathogenetic mechanism of viral infection, antiviral therapy and so on.

Peer review

There is interest in humanized rats and mice for studies of human metabolism and transplantation. In this paper rat livers are repopulated with human hepatocytes after chemical treatment with retrorsine and partial hepatectomy.

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BASIC RESEARCH

Preservation of non-heart-beating donor livers in extracorporeal liver perfusion and histidine-tryptophan-ketoglutarate solution

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before liver transplantation.

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Abstract

AIM: To compare the preservation of non-heart-beating donor (NHBD) livers in cold histidine-tryptophan-ketoglutarate (HTK) solution and extracorporeal liver perfusion (ECLP).

METHODS: Livers harvested from health pigs were stored for 10 h in cold HTK solution (group A, $n = 4$) or perfused with oxygenated autologous blood at body temperature (group B, $n = 4$). Both groups were then tested on the circuit for 4 h. Bile production, hemodynamic parameters, hepatocyte markers and reperfusion injury of extracorporeal livers were tested in each group. Liver tissues from each group were examined at the end of reperfusion.

RESULTS: At 1, 2, 3 and 4 h after reperfusion, bile production, hemodynamic parameters, hepatocyte markers and reperfusion injury of livers in group A were statistically different from those in group B ($P < 0.05$ or $P < 0.01$).

CONCLUSION: ECLP is better than HTK solution to preserve NHBD livers. ECLP can assess the graft viability

INTRODUCTION

Standard liver preservation involves flushing the liver *in situ* with an organ preservation solution [University of Wisconsin (UW) solution or histidine-tryptophan-ketoglutarate (HTK) solution] and its storage at 4°C for up to 15-18 h before transplantation^[1]. During the process, pre-preservation injury, cold-preservation injury, re-warming injury, and reperfusion injury may occur. The occurrence rate of primary non-function (PNF) ranges 2%-23%, which is a major cause of death in transplantation^[2]. Additionally, there is evidence that severe preservation injury is associated with increased liver graft rejection^[3].

The system of extracorporeal liver perfusion (ECLP), which was established by our research team, can perfuse the liver with an oxygenated perfusate supplemented with nutritional support at body temperature during the preservation period^[4]. Studies demonstrated that oxygenated perfusion is able to prevent adenosine tri-phosphate (ATP) loss during ischemia, and reset ATP levels after it^[5,6].

In this preclinical study, we compared standard cold storage of livers in HTK solution to normothermic, sanguineous perfusion over a 10 h preservation period in the pig liver model.

MATERIALS AND METHODS

Animals

Large white pigs weighing 20–25 kg were obtained from the Experimental Animal Center of Academy of Military Medical Sciences. All animals used in this study received humane care according to the National Guidelines for the Care of Animals in China. The pigs were housed at 23°C–25°C and fed with a standard food with free access to drinking water.

Equipments

Blood pump (JHBP-2000B) was purchased from Guangzhou Jihua Medical Instruments Company. Hollow fiber oxygenator 3381 and heat exchanger were produced by Medtronic, Inc. USA. Digital measuring and controlling heating water box (GKX21Cr) was a product of Shanghai Jinping Instrument and Meter Company, Shanghai, China. Premature infant incubator (KXK-5G) was made by the 7th Shanghai Medical Instrument Factory, Shanghai, China. Gathering blood container 2000 was purchased from Dongguan Kewei Medical Instrument Company, Guangzhou, China. Euro-Collins solution and HTK solution were from Koehler Chemie GmbH, Germany.

Experimental design

Livers were harvested from healthy pigs and preserved for 10 h in cold HTK solution (group A, $n = 4$) or perfused with oxygenated autologous blood at body temperature (group B, $n = 4$).

Reperfusion

The function of all harvested livers was tested after preservation. The ECLP system was used to perfuse the livers with whole blood at body temperature as a substitute for transplantation. The blood used to perfuse livers in group B was changed between the preservation and reperfusion phases and their physiological parameters were compared.

Operation

Animals were anesthetized with 4 mg/kg propofol (intravenous injection, i.v.) and their ear veins were cannulated. The animals were intubated with an extended endotracheal tube. Anesthesia was maintained with i.v. propofol (1 mg/kg per hour) and halothane inhalation *via* an extended endotracheal tube throughout the procedure. The artery and vein of cervix were inserted into two catheters for transfusion and collecting blood. A midline incision was made to enter the peritoneal cavity. The bile duct was divided and catheterized for bile juice drainage. Hepatic vessels were identified and isolated in a standard fashion. After the collection of autoblood, the portal vein and hepatic artery were catheterized and the inferior vena cava (IVC) was divided with a suite of catheters, then the whole liver was removed. Approximately 1000 mL of autoblood was collected into a reservoir bottle.

Perfusion

During the preparation of liver, the perfusion apparatus was assembled and primed with 1000 mL of pig blood (Figure 1). The perfusion circuit consisting of blood pump, oxygenator, gathering blood container, perfusion

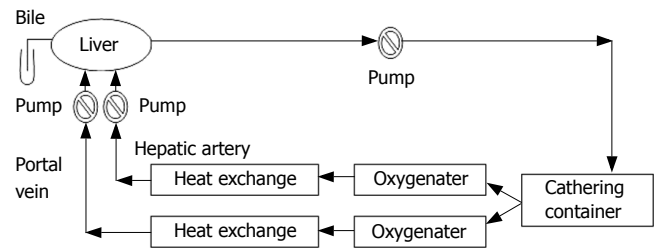


Figure 1 Hepatic circuit for extracorporeal liver perfusion.

Table 1 Bile production in liver of each group at different time points ($n = 4$, mean \pm SD)

Group	Bile production in liver (mL)			
	1 h	2 h	3 h	4 h
A	1.75 \pm 0.29	2.25 \pm 0.29	3.88 \pm 0.25	5.25 \pm 0.29
B	2.62 \pm 0.25 ^b	4.63 \pm 0.48 ^b	6.00 \pm 0.41 ^b	6.63 \pm 0.25 ^b

^b $P < 0.01$ vs group A.

tubing, gate clamp and heat exchanger was used to maintain the blood temperature at 39°C (normal temperature for a pig). Bottled oxygen was used and ratios were adjusted to maintain the physiological partial pressures. During priming of the circuit, pH, PaCO₂, and Ca²⁺ were adjusted to the normal physiologic range. In general, the perfusion circuit was supplemented with 20 mL 50% glucose and 20 U/heparin.

Assessment of liver function

The general conditions of liver were observed during perfusion. Bile production, hemodynamic parameters, hepatocyte markers and reperfusion injury of extracorporeal livers in each group were documented every hour during the reperfusion phase. At the end of each perfusion, the liver was sectioned and multiple random samples were assessed for histologic evaluation of reperfusion injury.

Statistical analysis

The data were analyzed using SPSS 11.0 statistical package. All values were presented as mean \pm SD. For a single comparison, the significance of differences between means was determined by Student's *t*-test. $P < 0.05$ was considered statistically significant.

RESULTS

Synthetic function

At 1, 2, 3 and 4 h after perfusion, bile production of livers in group A was statistically different from that in group B ($P = 0.0084, 0.0072, 0.0046, 0.0057$, respectively; Table 1).

Hemodynamic parameter

At 1, 2 and 3 h after perfusion, the pressure of hepatic artery in group A was statistically different from that in group B ($P < 0.05$), but at 4 h after perfusion, the pressure of hepatic artery in group A was not statistically different from that in group B. At 1 h after perfusion, the pressure

Table 2 Pressure of hepatic artery and portal vein in each group at different time points ($n = 4$, mean \pm SD)

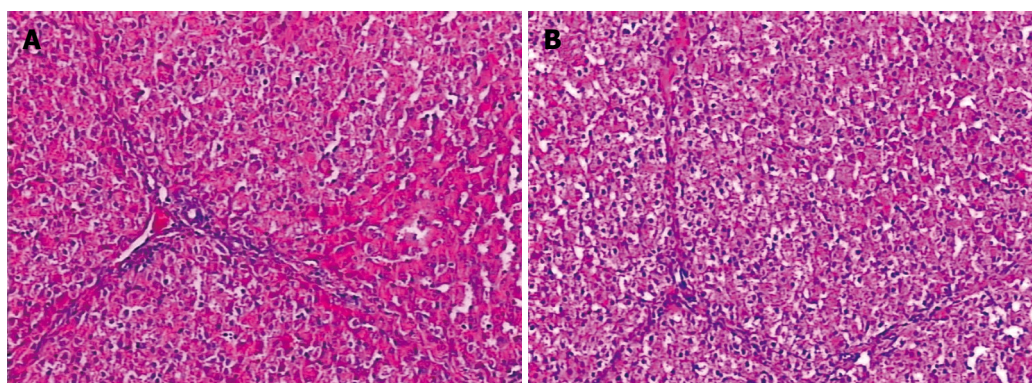
Group	Pressure of hepatic artery (mmHg)				Pressure of portal vein (mmHg)			
	1 h	2 h	3 h	4 h	1 h	2 h	3 h	4 h
A	101.13 \pm 1.65	98.75 \pm 1.94	93.75 \pm 1.19	90.50 \pm 1.58	8.25 \pm 0.65	7.50 \pm 0.41	7.00 \pm 0.41	6.63 \pm 0.25
B	97.75 \pm 1.44 ^a	94.50 \pm 1.08 ^a	91.25 \pm 1.04 ^b	89.88 \pm 1.11	7.50 \pm 0.41 ^a	7.13 \pm 0.48	6.75 \pm 0.29	6.50 \pm 0.41

^a $P < 0.05$, ^b $P < 0.01$ vs group A.Table 3 Level of ALT and LDH in perfusate of each group at different time points ($n = 4$, mean \pm SD)

Group	Level of ALT (U/L)				Level of LDH (U/L)			
	1 h	2 h	3 h	4 h	1 h	2 h	3 h	4 h
A	62.50 \pm 4.20	80.75 \pm 5.62	96.75 \pm 4.57	115.50 \pm 6.25	619.50 \pm 19.54	787.00 \pm 23.35	897.25 \pm 17.78	974.25 \pm 27.32
B	38.50 \pm 2.89 ^b	42.50 \pm 2.89 ^b	46.35 \pm 3.30 ^b	53.25 \pm 2.63 ^b	442.00 \pm 19.58 ^b	535.00 \pm 20.43 ^b	629.50 \pm 45.89 ^b	687.25 \pm 25.94 ^b

^b $P < 0.01$ vs group A.Table 4 Level of glucose in perfusate and oxygen consumption in livers of each group at different time points ($n = 4$, mean \pm SD)

Group	Level of glucose in perfusate (mmol/L)				Oxygen consumption in livers			
	1 h	2 h	3 h	4 h	1 h	2 h	3 h	4 h
A	619.50 \pm 19.54	787.00 \pm 23.35	897.25 \pm 17.78	974.25 \pm 27.32	269.50 \pm 12.45	234.50 \pm 13.48	216.00 \pm 7.75	192.25 \pm 8.50
B	442.00 \pm 19.58 ^b	535.00 \pm 20.43 ^b	629.50 \pm 45.89 ^b	687.25 \pm 25.94 ^b	244.00 \pm 8.44 ^b	267.00 \pm 7.07 ^b	258.00 \pm 4.97	251.25 \pm 5.74

^a $P < 0.05$, ^b $P < 0.01$ vs group A.Figure 2 Microstructure changes in hepatic cells (HE, $\times 200$) of group A (A) and group B (B).

of portal vein in group A was statistically different from that in group B ($P < 0.05$), but at 4 h after perfusion, the pressure of portal vein in group A was not statistically different from that in group C (Table 2).

Hepatocellular damage

A significant difference ($P < 0.01$) was found in alanine aminotransferase (ALT) between the two groups, which was increased to 115 IU/L in the cold-preserved livers. The corresponding value in the warm-preserved group never exceeded 55 IU/L. A similar change was seen in lactate dehydrogenase (LDH) release ($P < 0.01$), which was higher in cold-preserved livers (Table 3).

Metabolic function

A significant difference ($P < 0.01$) was found in glucose concentrations of the perfusate between the two groups. At 2, 3 and 4 h after perfusion, the rate of oxygen con-

sumption of livers in group A was statistically lower than that in group B ($P < 0.01$), but at 1 h after perfusion the rate of oxygen consumption of livers in group A was higher than that in group B ($P < 0.05$, Table 4).

Histological findings

Changes in microstructure of hepatic cells: The livers in group A revealed edema of hepatic cells, sporadic acidophilic degeneration, mild vacuolization and sinusoidal dilatation. The livers in group B showed slight edema and necrosis of few hepatic cells (Figure 2).

Changes in ultrastructure of hepatic cells: In group A, the karyotheca of hepatic cells was not clear, mitochondria swelled mildly, a few of crista vanished and endoplasmic reticulum expanded slightly. In group B, the karyotheca of hepatic cells was clear and regular, double nuclei were visible and endoplasmic reticulum was normal (Figure 3).

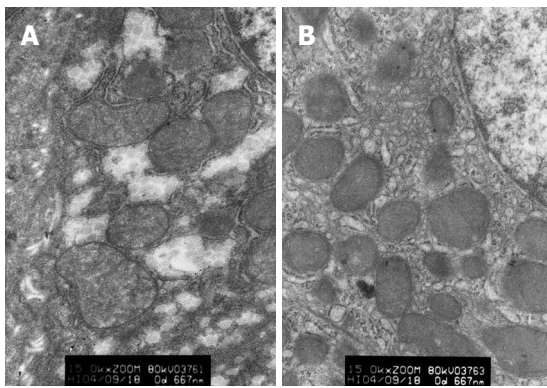


Figure 3 Ultrastructure changes in hepatic cells ($\times 15000$) of group A (A) and group B (B).

DISCUSSION

The progressively increases of patients with end stage liver disease is extending the waiting-list for liver transplantation, which, unfortunately, is not followed by a suitable increase in the number of donors^[7]. The shortage of donor organs has given rise to the interest in liver harvesting from NHBD, which can significantly reduce the large discrepancy between supply and demand for liver transplantation. However, NHBD livers suffer not only from warm ischemic injury but also from cold preservation and reperfusion injury^[8]. In this pathophysiological process, a number of cytokines, such as TNF- α , release with the activation of Kupffer cells and the obstruction of microcirculation. When blood perfusion is terminated during ischemic preservation, the supply of oxygen and nutrients is eliminated along with the vehicle for disposal of waste and metabolic by-products. During anoxia, ineffective anaerobic metabolism leads to depletion in energy stores, with a concomitant build-up of acidic by-products, which finally results in loss of transcellular electrolyte gradients, cell swelling, influx of free calcium, and subsequent activation of phospholipases. The breakdown of ATP during ischemia creates a setting for the production of reactive oxygen intermediates during reperfusion and a cascade of ischemic injury^[9-12].

During the first half of the twentieth century, Carrel^[13] perfused organs with normothermic, oxygenated serum at supraphysiological volumes and demonstrated gross viability for several days. ECLP has too many external constraints and has thus been largely abandoned. Research of ECLP has largely centered on the treatment of acute liver failure as a bridge to transplantation^[14-16]. The present study used the system of ECLP to perfuse NHBD livers using dual vessel normothermic sanguineous perfusion with oxygenated blood as the perfusate. The perfusate to the liver artery and portal vein can be oxygenated separately^[4]. Compared to the storage in cold HTK solution, the ECLP circuit acts not only as a method to store NHBD livers but as a substitute for transplantation, thus, the effect of two preservation methods can be evaluated. The outcome of studies can be influenced by many factors in a large animal transplantation model. If these factors are not considered, any conclusions drawn from this study can be attributed

directly to the preservation process.

The change in hemodynamic parameters reflects the condition of liver microcirculation^[17]. After 1 h reperfusion with the total hepatic blood flow maintained, the difference in the pressure of liver artery and observed in two groups, showing the different grade obstruction of liver microcirculation. When the reperfusion was continued, the pressure of liver artery and portal vein decreased gradually, while the pressure of portal vein remained unchanged in two groups, indicating that the liver microcirculation is improved. However, the condition of liver microcirculation in group B was better than that in group A. The glucose level in the perfusate represents the balance between supply and uptake of hepatocytes^[18], suggesting that the glucose level is an indirect measure of liver metabolism. It was reported that the functional state of an isolated liver can be measured from its ability to induce gluconeogenesis^[19]. The estimated elimination capacity of glucose concentration seems to reflect the metabolic capabilities during reperfusion. In the study, the glucose level of perfusate in group A was higher than that in group B, suggesting that the ability of liver to take up glucose is different in two groups. Although oxygen consumption might be reasonably assumed to be a measure of hepatocyte function, it fell consistently during perfusion. This was in contrast to other markers of liver function, particularly synthetic activity. Greater oxygen consumption was seen in livers of group A after 1 h reperfusion, which may be explained by the respiratory burst and subsequent oxygen debt that follows prolonged periods of cold ischemia and subsequent reperfusion. This was in contrast to the livers of group B, in which a stable level of oxygen consumption was observed. Bile production increased with time, which may reflect the consumption of substrate and its synthesizing function^[20,21].

How to use NHBD livers reasonably and effectively is the key question, which can make the transplanted liver function well and give the recipient a realistic chance of survival. At present, the criteria to decide whether the organ should be utilized or discarded almost depend on an overall judgment made on macroscopic appearance, perhaps with a biopsy. The ECLP system offers an opportunity to assess the viability of a liver to be used^[22,23], which may lead not only to a more rational use of the current donor pool but also to an accurate assessment of marginal organs^[24].

Although the technique of cold storage is effective and simple, it may have certain limitations in terms of ability to maintain a viable organ avoid of ischemic injury and preservation time. In addition, this technique does not allow for viability assessment, and therefore the use of marginal donor organs remains difficult. By simulating the natural physiological environment of the liver and providing oxygen and other substrates necessary for normal metabolism, ECLP may serve as an ideal organ preservation technique in the future^[25-27].

In conclusion, it is better to preserve NHBD livers in ECLP than in cold HTK solution in terms of synthetic and metabolic serum markers, hemodynamic parameters, and histological appearances.

COMMENTS

Background

To minimize the ischemia-reperfusion injury in non-heart-beating donor (NHBD) livers, we compared the preservation of NHBD livers in cold histidine-tryptophan-ketoglutarate (HTK) solution and extracorporeal liver perfusion (ECLP).

Research frontiers

Extracorporeal liver perfusion of NHBD livers can promote cellular recovery from warm ischaemic injury. The present study describes a method of normothermic extracorporeal perfusion by which the viability of livers can be maintained for at least 72 h.

Innovations and breakthroughs

This is probably the first study using the ECLP system with oxygenating blood. The ECLP circuit can be used not only as a method to store NHBD livers but as a substitute for transplantation, and therefore the effect of two preservation methods can be evaluated.

Applications

The findings in this study support that normothermic, sanguineous and oxygenated ECLP is better than HTK solution to preserve NHBD liver.

Peer review

The design of this study was rational and reliable. The statistical methods used were appropriate. The results were sufficient to draw the conclusions. The discussion was well organized and rational.

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Effect of Chaqinchengqi decoction on sarco/endoplasmic reticulum Ca^{2+} -ATPase mRNA expression of pancreatic tissues in acute pancreatitis rats

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Abstract

AIM: To investigate the effect of Chaqinchengqi decoction (CQCQD) on sarco/endoplasmic reticulum Ca^{2+} -ATPase (*SERCA*) mRNA expression of pancreatic tissues in acute pancreatitis (AP) rats.

METHODS: Thirty Sprague-Dawley (SD) rats were randomized into control group, AP group and CQCQD group ($n = 3 \times 10$). The rats in the CQCQD group were intragastrically administered with CQCQD (10 mL/kg every 2 h) after induction of AP by intraperitoneal injection of caerulein (50 $\mu\text{g}/\text{kg} \cdot \text{h} \times 5$) within 4 h. At 6 h after the induction of AP model, pancreatic tissues were collected for the pathological observation, mRNA extraction for determination of *SERCA1* and *SERCA2* mRNA expression or pancreatic acinar cell isolation for measurement of fluorescence intensity (FI) of intracellular calcium ion concentration $[\text{Ca}^{2+}]_i$.

RESULTS: There was no expression of pancreatic *SERCA1* mRNA in the control group and the AP group. The expression of pancreatic *SERCA2* mRNA in the AP group was down-regulated (expression ratio = 0.536; $P = 0.001$) compared with the control group, while that in the CQCQD group was up-regulated (expression ratio

= 2.00; $P = 0.012$) compared with AP group. The FI of intracellular $[\text{Ca}^{2+}]_i$ of pancreatic acinar cells in the AP group (138.2 ± 23.1) was higher than the C group (111.0 ± 18.4) and the CQCQD group (118.7 ± 15.2) ($P < 0.05$) and the pancreatic pathological score in the CQCQD group was lower than that in the AP group (5.7 ± 1.9 vs 9.2 ± 2.7 , $P < 0.05$).

CONCLUSION: CQCQD can up-regulate the expression of *SERCA2* mRNA of pancreatic tissues, reduce intracellular calcium overload and relieve pancreatic tissue lesions.

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Key words: Chaqinchengqi decoction; Pancreatitis; Calcium overload; Sarco/endoplasmic reticulum Ca^{2+} -ATPase

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INTRODUCTION

Acute pancreatitis (AP) is a potentially lethal disorder with no specific therapeutic options^[1-5]. The major obstacle to the development of therapies is our limited understanding of the pathogenesis of AP. Despite a number of theories have been proposed to explain the pathogenesis from various aspects, but there are still controversies about the mechanism of the disorder. Since Ward^[6] firstly proposed that calcium overload in pancreatic acinar cells should be a “trigger point” of AP, intracellular calcium overload has been generally accepted to play a crucial role in the occurrence and deterioration of AP^[7].

The increased intracellular free calcium ion concentration ($[\text{Ca}^{2+}]_i$) is believed to originate from the influx of extracellular Ca^{2+} and the release of Ca^{2+} of intracellular Ca^{2+} stores^[8]. In AP, the quantity and/or activity of plasma membrane Ca^{2+} -ATPase (PMCA) and/or sarco/endoplasmic reticulum Ca^{2+} -ATPase (SERCA)

were decreased, the intracellular Ca^{2+} was not pumped out of cell or back into Ca^{2+} stores in time^[9]. But the exact mechanism of intracellular calcium overload in AP remains unclear. Therefore, a better elucidation would improve the therapeutic strategies of this disease.

Chaiqinchengqi decoction (CQCQD) has been proved to be effective in the treatment of AP^[10]. Our previous experiments found that CQCQD inhibited the elevation of $[\text{Ca}^{2+}]_i$ and had a protective effect on pancreatic acinar cells in AP rats^[11]. In the present study, we aim to explore the mechanism of intracellular calcium overload by determining the *SERCA* mRNA expression changes of pancreatic tissues in AP rats and further to find out the therapeutic mechanisms of CQCQD in AP rats.

MATERIALS AND METHODS

Animal

Thirty male Sprague-Dawley (SD) rats weighing 250-300 g were purchased from the Experimental Animal Center of West China Center of Medical Sciences of Sichuan University. All animals were kept at constant room temperature in a 12-h light/dark cycle with free access to standard chow and water. All animals were adjusted to laboratory conditions for 1 wk and a 12-h fast with free access to drinking water before the experiments. This study was approved by and conducted under the guidelines of Animal Use and the Committee of Scientific Research of West China Hospital of Sichuan University.

Reagents

Caerulein was purchased from Sigma Co. (USA), trizol and diethyl pyrocarbonate (DEPC) were from Invitrogen Company (USA), hexanucleotide random primer and Superscript II RNase H-reverse transcriptase (RNase H-RT) was from Life Technologies, dNTPs and the RNAase inhibitor were from Takara Co. (Japan), and Taq DNA polymerase was from Promega (USA). Primers for *SERCA1* and *SERCA2* gene were designed according to the method reported previously. Primers for *SERCA1*, *SERCA2* and *GAPDH* genes were synthesized by Shanghai Biotechnology Co. Ltd. (Table 1). Dulbecco's modified Eagle's medium (DMEM) is a product of GIBCO BRL and preoxygenated for 30 min before use. Collagenase P was purchased from Roche Applied Science. Fluo-3/AM was a product of Molecular Probes Co, USA. All other reagents were of the highest purity available and were purchased from Sigma unless indicated otherwise.

Preparation of CQCQD

The Chinese medicinal herbs in CQCQD provided by Chinese Herbs Pharmacy of West Chinese Hospital of Sichuan University included Chaihu (*Radix Bupleuri*) 15 g, Huangqin (*Radix Scutellariae*) 15 g, Houpo (*Cortex Magnoliae Officinalis*) 15 g, Zhishi (*Fructus Aurantii Immaturus*) 15 g, Yinchén (*Herba Artemisiae Scopariae*) 15 g, Zhizi (*Fructus Gardeniae*) 20 g, Dahuang (*Radix et Rhizoma Rhei*) 15 g and Mangxiao (*Natrii Sulfas*) 10 g. The decoction was made into 200 mL juice and then into lyophilized powder. Before experiment, the lyophilized powder of CQCQD was prepared to a concentration of 2 g/mL of crude herbs.

Animal grouping and procedure

SD rats were randomized into control group (C group, $n = 10$), AP group ($n = 10$) and CQCQD group ($n = 10$). AP model was induced by intraperitoneal injection of caerulein ($50 \mu\text{g}/\text{kg} \cdot \text{h} \times 5$) within 4 h^[12]. Rats in the C group were administered with the same volume of physiological saline. After the induction of AP, the rats in the CQCQD group were injected orally into the stomach with CQCQD ($10 \text{ mL}/\text{kg} \cdot 2 \text{ h}$), and other two groups were intragastrically administered with the same volume of physiological saline as CQCQD group. At 6 h after the induction of AP, the rats were anesthetized with intraperitoneal sodium pentobarbital ($40 \text{ mg}/\text{kg}$), the abdomen was opened and the pancreatic tissues were rapidly collected for pathological examination, mRNA extraction or cell isolation for measurement of $[\text{Ca}^{2+}]_i$. The rats were sacrificed by exsanguinations after experiments.

Pathological examination and scoring of pancreatic tissues

After removal of the pancreatic tissues, the sections of samples were fixed in 4% neutral buffer formaldehyde, embedded with paraffin wax, cut into slices, and stained with Hematoxylin-Eosin (HE) and observed under light microscopy. Pathological grading and scoring criteria are shown in Table 2^[13-15]. For each pathological section, 10 visual fields under a high-power microscope (HE, $\times 400$) were randomly selected and scored by one pathologist. The mean score of the 10 visual fields of one pathological section was calculated as the pathological score.

Pancreatic acinar cell isolation and $[\text{Ca}^{2+}]_i$ measurement

Pancreatic tissue was quickly removed and immediately transferred to iced DMEM. Collagenase P ($0.3 \text{ g}/\text{L}$) in 2.5 mL DMEM containing $2 \text{ g}/\text{L}$ bovine serum albumin (BSA) and $0.1 \text{ g}/\text{L}$ soybean trypsin inhibitor (SBTI) was infiltrated into the tissue with a 5 mL syringe. The tissue was minced into small fragments and digested in Collagenase P solution at 37°C in a shaking water bath for $3 \times 15 \text{ min}$ ($120 \text{ cycles}/\text{min}$), dispersed with a plastic pipette, filtered through a nylon mesh (150 meshes), and layered into DMEM containing 4% BSA. The acinar cells were centrifuged at $400 \text{ r}/\text{min}$ for 3 min for three times in fresh DMEM medium containing $2 \text{ g}/\text{L}$ BSA and $0.1 \text{ g}/\text{L}$ SBTI and resuspended before use.

According to the previously reported methods^[16], the pancreatic acinar cells were loaded with $5 \mu\text{mol}/\text{L}$ Fluo-3/AM and 0.01% Pluronic F-127 in darkness for 30 min at 37°C and observed under Leica TCS-sp2 laser scanning confocal microscopy (LSCM). Twenty different cells in each visual field were randomly selected. The cells loaded with Fluo-3/AM were excited at a wavelength of 488 nm and the emitted fluorescence was detected at 488 nm and 560 nm. The relative fluorescence was analyzed using fluorescence quantitative analysis software and the intracellular calcium fluorescence intensity (FI) was presented as intracellular $[\text{Ca}^{2+}]_i$.

Reverse transcription and polymerase-chain-reaction (RT-PCR)

Pancreatic tissues in each group was quickly removed and

Table 1 Probe, primer and product (bp) in RT-PCR

Genes	Probes	Upstream primers	Downstream primers	Product (bp)
<i>SERCA1</i>	5'-CGATGTCCCGAGCCTTGATCC-3'	5'-GGTTTGGCAGGAACGGAAT-3'	5'-GGTGGATTGATGGAGAGGAT-3'	199
<i>SERCA2</i>	5'-CACACTCTTTCTGCTGCTCG-3'	5'-ATGAACCTGAAATGGGCAAG-3'	5'-GGAACCTTGTGACCAACAGCA-3'	115
<i>GAPDH</i>	5'-FAM-ACCACAGTCCATGCCATCAC-TAMRA-3'	5'-CCTCAAGATGTGTCAGCAAT-3'	5'-CCATCCACAGTCTTCTGAGT-3'	141

SERCA: Sarco/endoplasmic reticulum Ca^{2+} -ATPase; *GAPDH*: Glyceraldehyde-3-phosphate dehydrogenase.

Table 2 Pathological scoring criteria for pancreatic tissues of AP rats

Pathological changes	Scores
Edema	
Focal expansion of interlobular septa	1
Same as 1 + diffuse expansion of interlobar; Septa/diffuse expansion of interlobar septa	2
Same as 2 + expansion of interacinar septa	3
Same as 3 + expansion of intercellular spaces	4
Inflammation and perivascular infiltrate	
2-10 intralobular or perivascular leukocytes/HPF	1
11-20 intralobular or perivascular leukocytes/HPF	2
21-30 intralobular or perivascular leukocytes/HPF	3
> 30 leukocytes/HPF or confluent microabscesses	4
Acinar necrosis	
Diffuse occurrence of 1-4 necrotic cells/HPF	1
Diffuse occurrence of 5-10 necrotic cells/HPF	2
Diffuse occurrence of 11-16 necrotic cells/HPF (foci of confluent necrosis)	3
> 16 necrotic cells/HPF (Extensive confluent necrosis)	4
Hemorrhage and fat necrosis	
1-2 focus	1
3-4 focus	2
5-6 focus	3
> 7 focus	4

immediately transferred into liquid nitrogen. Total RNA of pancreatic tissue was extracted using Trizol reagent. The integrity of the total RNA was confirmed by agarose gel electrophoresis. Quantity and purity of the total RNA were determined by an ultraviolet spectrophotometer, and RNA concentration of the sample was calculated.

The total RNA was reverse-transcribed using hexanucleotide random primers with RNase H-RT. The cDNA was amplified as a template for subsequent PCR using *Taq* DNA polymerase in a Perkin Elmer Cetus DNA thermocycler. PCR reaction system included 5 μL cDNA-RT product, 3 μL 10 \times PCR buffer, 3 μL MgCl_2 of 25 mmol/L, 0.36 μL dNTP of 25 mmol/L, 1 μL upstream primer of 10 $\mu\text{mol/L}$, 1 μL downstream primer of 10 $\mu\text{mol/L}$, 0.6 μL probe of 10 $\mu\text{mol/L}$, 0.3 μL *Taq* polymerase of 5 U/ μL , and 15.74 μL deionized double-distilled water. PCR cycling conditions were as follows: initial denaturation at 94°C for 2 min, followed by 45 cycles of 94°C for 20 s, 56°C for 30 s and 60°C for 40 s.

In this study, a negative control with the same volume of deionized double-distilled water instead of cDNA was used and amplified to control DNA contamination, and a positive control with the same volume of the cDNA of skeletal muscle of rat instead of cDNA of pancreatic tissues. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*)

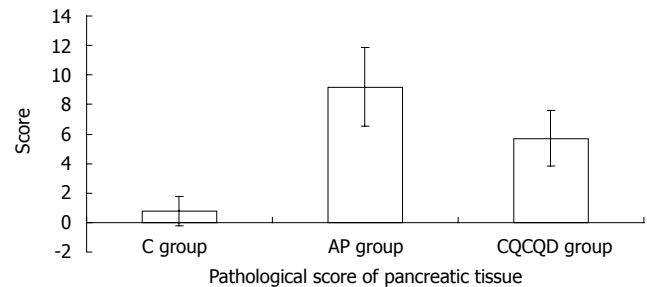


Figure 1 Scores of pancreatic tissues in three groups ($n = 10$, mean \pm SD). The pathological score of pancreatic tissues was the highest in the AP group (9.2 ± 2.7) and followed by CQCQD group (5.7 ± 1.9) and C group (0.8 ± 1.0), ($P < 0.05$).

gene was used as internal housekeeping gene. The amplified DNAs were resolved by agarose gel electrophoresis and stained with ethidium bromide. The bands were visualized and photographed under ultraviolet light.

Statistical analysis

Data were expressed as mean \pm SD. Data in normal distribution were analyzed using analysis of variance; data in abnormal distribution was analyzed using Wilcoxon rank sum test. The Relative Expression Software Tool was employed for statistical analysis of gene expression^[17,18]. $P < 0.05$ was considered statistically significant.

RESULTS

Pathological scoring of pancreatic tissues

The pathological score of pancreatic tissue in the AP group was the highest (9.2 ± 2.7) and followed by the CQCQD group (5.7 ± 1.9) and the C group (0.8 ± 1.0) ($P < 0.05$), (Figure 1).

Intracellular $[\text{Ca}^{2+}]_i$ of pancreatic acinar cells

The FI of $[\text{Ca}^{2+}]_i$ pancreatic acinar cells in the AP group was higher than that in the C group (138.2 ± 23.1 vs 111.0 ± 18.4) or CQCQD group (138.2 ± 23.1 vs 118.7 ± 15.2), ($P < 0.05$), but there was no statistical difference between the C group and CQCQD group in $[\text{Ca}^{2+}]_i$ ($P > 0.05$), (Figure 2).

SERCA1 mRNA expression

Neither the pancreatic tissues of normal rats nor that of AP rats expressed *SERCA1* mRNA. Skeletal muscle, however, expressed *SERCA1* mRNA as the positive control, shown by the electrophoresis of PCR products in Figure 3.

SERCA2 mRNA expression

Cycle threshold (Ct) value of *GAPDH* and *SERCA2*

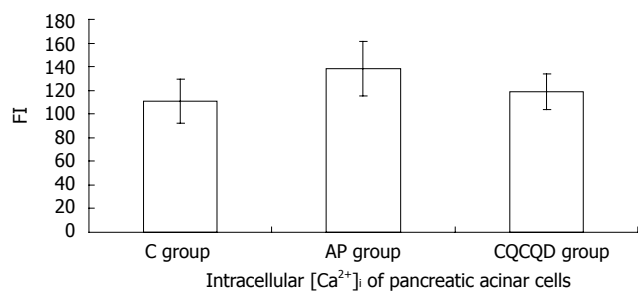


Figure 2 Intracellular $[Ca^{2+}]_i$ of pancreatic acini ($n = 10$, mean \pm SD). The fluorescence intensity (FI) of intracellular $[Ca^{2+}]_i$ of pancreatic acinar in AP group (138.2 ± 23.1) was higher than C group (111.0 ± 18.4) and CQCQD group (118.7 ± 15.2 , $P < 0.05$), but there was no statistical difference between the C group and the CQCQD group ($P > 0.05$).

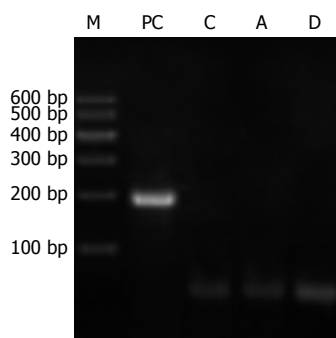


Figure 3 Agar gel electrophoresis photograph of PCR products of *SERCA1* mRNA. The PCR products of sarcoplasmic reticulum Ca^{2+} -ATPase (*SERCA1*) mRNA was 199 bp. M: Marker; PC: Positive control with skeletal muscle; C: Control group; A: Acute pancreatitis group; D: Acute pancreatitis group treated with Chaiqinchengqi decoction.

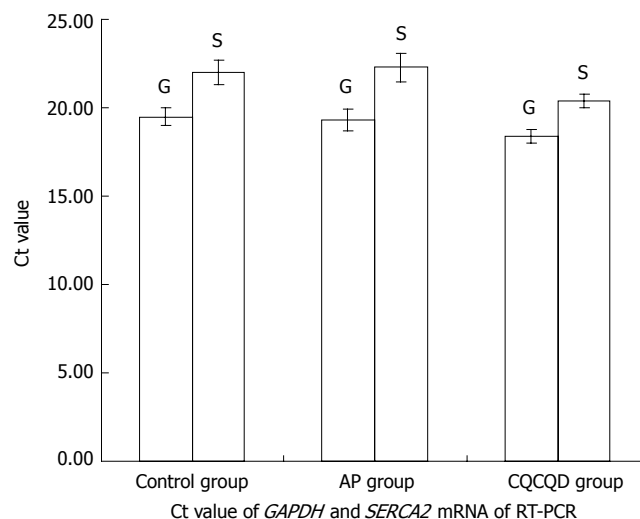


Figure 4 Ct value of *GAPDH* and *SERCA2* mRNA of RT-PCR. Ct: Cycle threshold; G: Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), the internal housekeeping; S: Sarco/endoplasmic reticulum Ca^{2+} -ATPase 2 (*SERCA2*).

mRNA in the three groups is shown in Figure 4 and the agar gel electrophoresis photograph of PCR products of *SERCA2* mRNA is shown in Figure 5. The expression of *SERCA2* mRNA was highest in the C group, and lowest in the AP group, and moderate in the CQCQD group. Compared with the C group, the expression of *SERCA2* mRNA in the AP group was decreased with an expression ratio of 0.536 ($P = 0.001$). Compared with the AP group, the expression of *SERCA2* mRNA in the CQCQD group was increased with an expression ratio of 2.000 ($P = 0.012$).

DISCUSSION

Intracellular calcium overload of pancreatic acinar cells has been confirmed in experimental acute pancreatitis^[19-23] and extensively accepted to be an important triggering and exacerbating mechanism of AP^[6,7,24,25]. Ca^{2+} -ATPase plays a key role in the mechanism of intracellular calcium overload of pancreatic acinar cells. Qiu Y *et al*^[9] found that the activity of intracellular Ca^{2+} -Mg²⁺-ATPase was decreased in AP rats.

As it is known, Ca^{2+} in cytoplasm of pancreatic acinar cells during calcium overload mainly originates from the release of intracellular calcium stores^[26,27]. In pancreatic acinar cells, Ca^{2+} released from the store is mainly resequenced into the stores by SERCA and only partly expelled into the extracellular space by PMCA^[28]. There are three isoforms of SERCA, including SERCA1, SERCA2 and SERCA3 in rats, in which the expressions of *SERCA1* and *SERCA2* mRNA were found in pancreatic tissues. Therefore, intracellular Ca^{2+} -ATPase activity was also affected by the change of mRNA expression of the isoforms.

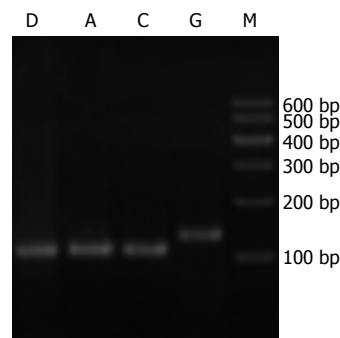


Figure 5 Agar gel electrophoresis photograph of PCR products of *SERCA2* mRNA. D: Acute pancreatitis group treated with Chaiqinchengqi decoction; A: Acute pancreatitis group; C: Control group; G: Internal housekeeping gene (*GAPDH* gene); M: Marker.

With the consideration that SERCA is the major contributor to maintenance of Ca^{2+} homeostasis and it may be affected by the isoforms of SERCA, a decreased quantity or activity of SERCA isoform would be expected to induce intracellular calcium overload and subsequently cause AP^[29-31] and the rising $[Ca^{2+}]_i$ of pancreatic acinar cells positively correlated to the severity of pancreatic pathology^[32,33]. To explore the mechanism of intracellular calcium overload in AP rats, we studied the change of *SERCA1* and *SERCA2* mRNA expressions by real-time RT-PCR. We found that the *SERCA1* mRNA did not express in pancreatic tissues of normal rats as well as AP rats with the positive control of rat skeletal muscle; the *SERCA2* mRNA expression of pancreatic tissues was down-regulated with remarkable elevation of intracellular $[Ca^{2+}]_i$ and pathological score of pancreatic tissues in AP rats. The results suggested that down-regulated *SERCA2* mRNA expression might take part in the pathogenesis of AP by decreasing the activity of SERCA and increasing $[Ca^{2+}]_i$ of pancreatic acinar cells in AP rats.

Chinese medicine CQCQD, modified from Dachengqi decoction, is an effective compound for the treatment of AP^[10]. It has been proved to improve systemic inflammatory response syndrome in acute necrotizing pancreatitis through the cholinergic anti-inflammatory pathway^[34]. We have also found that the protective effect

of CQCQD on pancreatic acinar cells was related to the inhibited exocrine of digestive enzymes and reduced elevation of $[Ca^{2+}]_i$ ^[11]. Chinese herbs in CQCQD including *Herba Artemisiae Scopariae*, Gardenia and rhubarb may have the activity of calcium channel blockers. Gardenia was found to inhibit the decline of activity of $Na^+-K^+-ATPase$ and PCMA in AP^[9]. Our study showed that in AP rats after treatment with CQCQD, *SERCA2* mRNA expression in pancreatic tissues was up-regulated, and intracellular $[Ca^{2+}]_i$ of pancreatic acinar cells and pathological score of the pancreatic tissues were decreased. The result indicates that CQCQD might protect pancreatic tissues from injuries by increasing SERCA activity, reducing calcium overload in pancreatic acinar cells and relieving the pathological severity.

In conclusion, the down-regulated expression of pancreatic *SERCA2* mRNA may be involved in the pathogenesis of intracellular calcium overload in AP. CQCQD has a protective effect on pancreatic tissues by increasing the expression of *SERCA2* mRNA and relieving intracellular calcium overload.

COMMENTS

Background

The pathogenesis of acute pancreatitis (AP) has not been fully elucidated up to date, whereas intracellular calcium overload of pancreatic acinar cells has been accepted as the pivot in triggering and deterioration of AP. The increased intracellular free calcium ion concentration ($[Ca^{2+}]_i$) is believed to originate from the influx of extracellular Ca^{2+} and the release of Ca^{2+} of intracellular Ca^{2+} stores. Despite of those theories proposed, the exact mechanism of intracellular calcium overload still remains obscure.

Research frontiers

This pilot animal experiment has found Chaiqinchengqi decoction (CQCQD), which is an effective herbal prescription in the treatment of AP, has a protective effect on pancreatic acinar cells in AP rats by inhibiting the elevation of $[Ca^{2+}]_i$. Advanced researches are needed to explore its mechanism.

Innovations and breakthroughs

Sarco/endoplasmic reticulum Ca^{2+} -ATPase (SERCA) has been proved to play a key role in the mechanism of intracellular calcium overload of pancreatic acinar cells, but the effect of CQCQD is not available in published studies. The authors innovatively studied the effect of CQCQD on *SERCA* mRNA expression of pancreatic tissue in AP rats. The result reveals for the first time that CQCQD up-regulated *SERCA* mRNA expression and relieved intracellular calcium overload to protect pancreatic tissues. Thus, this study elucidated the mechanism of CQCQD in the treatment of AP from genetic point of view.

Applications

As the efficacy of CQCQD has been proved in clinical trails, the present study intensively ratified the protective effect on pancreatic tissues in animal experiment. Moreover, the study in genetic mechanism of CQCQD in inhibiting intracellular calcium overload may light up advanced studies on calcium overload in the treatment of AP.

Terminology

Acute pancreatitis (AP) is a common acute abdominal disorder, caused by the unregulated activation of enzyme within pancreatic acinar cells and the autodigestion of the gland leading to inflammation of pancreas. Calcium overload is intracellular $[Ca^{2+}]_i$ abnormally increases which may subsequently lead to cell and tissue damages. Chaiqinchengqi decoction (CQCQD) is a herbal prescription comprising 8 Chinese herbals of Chaihu, Huangqin, Houpo, Zhishi, Zhizi and Mangxiao with the main therapeutic effect of clearing heat to detoxification and purging the bowel to restore the motility, which is a basic compound formula commonly used in the treatment of AP. Sarco/endoplasmic reticulum Ca^{2+} -ATPase

(SERCA) is an enzyme in arco/endoplasmic reticulum which in physiological conditions utilizes ATP as the energy to pump Ca^{2+} back to intracellular calcium stores to maintain intracellular calcium concentration in a normal range.

Peer review

This is a pilot research that focuses on the value of herbal medicine in reducing the severity of AP and exploring the mechanism on relieving intracellular calcium overload. The results of the study extend our understanding in the treatment mechanism of Chinese herbal prescription for AP and have an edificatory value to the studies in this field. Further researches such as protein expression are needed to explain its mechanism.

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Vascular endothelial growth factor attenuates hepatic sinusoidal capillarization in thioacetamide-induced cirrhotic rats

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Abstract

AIM: To investigate the effect of vascular endothelial growth factor (VEGF) transfection on hepatic sinusoidal capillarization.

METHODS: Enhanced green fluorescent protein (EGFP)/VEGF transfection was confirmed by immunofluorescence microscopy and immunohistochemistry both in primary hepatocytes and in normal liver. Cirrhotic rats were generated by thioacetamide (TAA) administration and then divided into a treatment group, which received injections of 400 µg of plasmid DNA encoding an EGFP-VEGF fusion protein, and a blank group, which received an equal amount of normal saline through the portal vein. The portal vein pressure was measured in the normal and cirrhotic state, in treated and blank groups. The average number of fenestrae per hepatic sinusoid was determined using transmission electron microscopy (TEM), while the relative abundance of VEGF transcripts was examined by Gene array.

RESULTS: Green fluorescent protein was observed in the cytoplasm of liver cells under immunofluorescence microscopy 24 h after transfection with EGFP/VEGF plasmid *in vitro*. Staining with polyclonal antibodies against VEGF illustrated that hepatocytes expressed

immunodetectable VEGF both *in vitro* and *in vivo*. There were significant differences in the number of fenestrae and portal vein pressures between normal and cirrhotic rats (7.40 ± 1.71 vs 2.30 ± 1.16 and 9.32 ± 0.85 cmH₂O vs 17.92 ± 0.90 cmH₂O, $P < 0.01$), between cirrhotic and treated rats (2.30 ± 1.16 cmH₂O vs 4.60 ± 1.65 and 17.92 ± 0.90 cmH₂O vs 15.52 ± 0.93 cmH₂O, $P < 0.05$) and between the treatment group and the blank group (4.60 ± 1.65 cmH₂O vs 2.10 ± 1.10 cmH₂O and 15.52 ± 0.93 cmH₂O vs 17.26 ± 1.80 cmH₂O, $P < 0.05$). Gene-array analysis revealed that the relative abundance of transcripts of VEGF family members decreased in the cirrhotic state and increased after transfection.

CONCLUSION: Injection of a plasmid encoding VEGF through the portal vein is an effective method to induce the formation of fenestrae and decrease portal vein pressure in cirrhotic rats. Therefore, it may be a good choice for treating hepatic cirrhosis and portal hypertension.

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Key words: Liver cirrhosis; Hepatic sinusoid capillarization; Fenestrae; Vascular endothelial growth factor; Transmission electrical microscopy; Ultrastructure; Gene array

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INTRODUCTION

Fenestrae are described as membrane-bound cytoplasmic holes with an average diameter of about 110 nm in transmission electrical microscopy (TEM) by Wisse in 1970^[1,2]. Liver sinusoidal endothelial cells (LSECs) possess open fenestrae that perforate the hepatic endothelial lining, but lack a basal lamina. Fenestrae, vesicles and channels together control the bulk of trans-endothelial transport between blood and tissues^[3]. Structural integrity of the

fenestrated sinusoidal liver endothelium is believed to be essential for the maintenance of the normal exchange of fluids, solutes, particles and metabolites between the sinusoidal blood and hepatocytes^[4,5]. Although there are various causes and morphologies of hepatic cirrhosis, all forms of cirrhosis are characterized by a defenestrated sinusoidal endothelium and the presence of a subendothelial basement membrane^[6-9]. It has been demonstrated that the disappearance of the normal filtration barrier in cirrhotic livers results in an impaired bidirectional exchange between the sinusoidal blood and parenchymal cells^[10]. As a result, capillarization of the sinusoidal endothelium may be a major contributor to hepatic failure in patients with cirrhosis.

Vascular endothelial growth factor (VEGF), which plays a role in regulating vasculogenesis, induces angiogenesis and endothelial cell proliferation. In recent years, it has been proven that VEGF is relevant to the increased number of fenestrae and endothelial permeability in endothelial cells^[10-13] and renal glomerulus^[14]. Injection of VEGF-D plasmid into both normal and ischemic rat liver resulted in an increased number of new capillaries around hepatic sinuses^[15]. Many studies have shown that, in the state of hepatic fibrosis and cirrhosis, both in patients and experimental models, VEGF is increased with harmful effects^[16,17]. By contrast, other studies have shown decreased expression of VEGF in cirrhotic patients, and suggested that VEGF makes a helpful contribution^[18,19]. After hepatectomy in cirrhotic rats, VEGF was found to effectively promote liver regeneration^[20,21]. Moreover, human urokinase-type plasminogen activator gene administration via an adenoviral (Ad)-vector induced cirrhosis regression and ameliorated hepatic dysfunction with up-regulation of VEGF in a model of experimental liver cirrhosis^[22,23].

Because defenestration and basement membrane formation result in a disordered exchange between the sinusoidal blood and hepatocytes, it is necessary to restore the function of liver sinusoidal endothelial cells in order to reverse cirrhosis. VEGF provides the perfect means to achieve this, because of it promotes fenestration and permeability. On account of these ideas, we studied the effects of VEGF transfection in cirrhotic rat livers.

MATERIALS AND METHODS

EGFP/VEGF transfection of hepatocytes

EGFP/VEGF was obtained from the Medical School of Shandong University (Jinan, China). Human VEGF-D cDNA was inserted into pEGFP-N1 plasmid between the constitutive cytomegalovirus promoter (pCMV) and enhanced green fluorescent protein (EGFP) to produce a plasmid encoding an EGFP-VEGF fusion protein (EGFP/VEGF plasmid), which was used as a tool to express VEGF consistently. EGFP was used to detect expression of the plasmid, and the expression of VEGF was detected by immunohistochemistry.

Primary hepatocytes were isolated from the livers of male Wistar rats (150-200 g) by collagenase perfusion as previously described^[24]. Cells were plated on collagen-

coated 6-well plates at a density of 3×10^5 cells/well in Williams' medium E supplemented with 10% fetal bovine serum, 2 mmol/L L-glutamine, 1% penicillin/streptomycin, and 100 nmol/L dexamethasone. The cells were incubated in 5% CO₂ at 37°C to facilitate attachment, and the medium was exchanged after 4 h with serum-free Williams' medium E supplemented with 2 mmol/L L-glutamine, 1% penicillin/streptomycin, and 100 nmol/L dexamethasone. After overnight incubation in 5% CO₂ at 37°C, the medium was exchanged with serum-free Williams' medium E supplemented with 2 mmol/L L-glutamine and 1% penicillin/streptomycin. The EGFP/VEGF plasmid (50 µg) was added to the culture medium and the cells were incubated for 24 h. We investigated 5 dishes for each treatment in the present study.

Immunofluorescence microscopy and immunohistochemistry

Immunofluorescence microscopy was used to visualize the distribution of EGFP in hepatocytes. At the same time, immunocytochemical staining of VEGF was performed. Cultured hepatocytes were washed with phosphate buffered saline (PBS) 3 times, and fixed with acetone at room temperature for 10 min. Then, they were blocked with goat serum and permeabilized for 30 min with 0.1% Triton X-100 in PBS containing 1% bovine serum albumin (BSA). The cells were incubated overnight with rabbit anti-human VEGF polyclonal antibody (Santa Cruz) at a 1:50 dilution at 4°C. Subsequently, they were incubated with tetramethylrhodamine isomer R1-conjugated swine anti-rabbit IgG (Santa Cruz) at room temperature for 1 h. The distribution of VEGF was visualized by light microscopy.

Transfection of normal liver

Twenty healthy male Wistar rats, weighing 200-220 g, bought from the experimental animal center of Shandong Agricultural Research Center (Jinan, China) were divided equally into two groups. In the study group, each rat received an injection of 400 µg of the EGFP/VEGF plasmid through the portal vein. In the control group, each rat received an equal amount of normal saline in the same manner. All rats were sacrificed eight days after the operation. Liver samples were collected and fixed in 10% neutral-buffered formalin, embedded in paraffin and cut into 4-6 µm sections.

Immunohistochemistry

Immunohistochemistry was performed according to Hong-Lei Weng^[25]. The integral score method was used to evaluate the positive cell distribution and intensity^[26]. First, a proportion score was assigned, which represented the estimated proportion of positive-staining cells (0, none; 1, 0 to 1/100; 2, 1/100 to 1/10; 3, 1/10 to 1/3; 4, 1/3 to 2/3; and 5, 2/3). Next, an intensity score was assigned, which represented the average intensity of positive cells (0, none; 1, weak, 2, intermediate; and 3, strong). The proportion and intensity scores were then added to obtain a total score, which ranged from 0 to 8. Then the score was compared between the study and control groups.

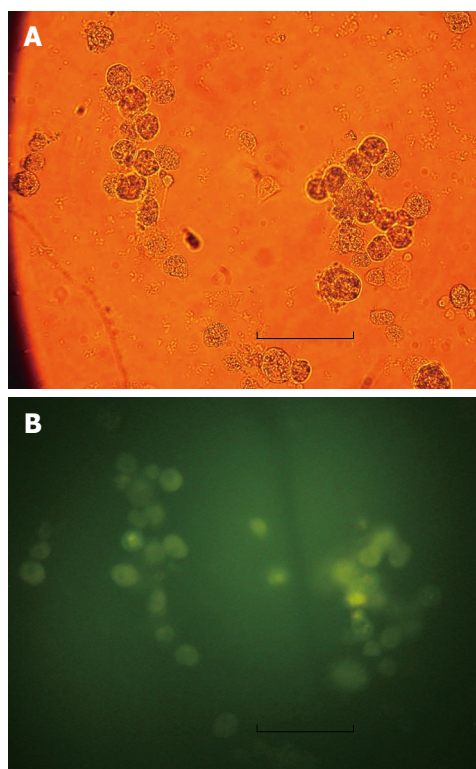


Figure 1 Hepatocytes incubated with EGFP/VEGF plasmid for 24 h. **A:** Light microscopy: no green fluorescent protein was found. Bar denotes 30 µm; **B:** Immunofluorescence microscopy: green fluorescent protein expression. Bar denotes 30 µm.

Transfection of cirrhotic liver

The portal vein pressures of 40 normal Wistar rats were measured and hepatic specimens were taken. Then, the rats received an oral administration of 0.03% thioacetamide (TAA) solution instead of feed water. After 5 wk, the concentration of TAA was increased to 0.04%. Ten wk later, 26 cirrhotic rats were identified and randomly divided into a treatment group and a blank group. The portal vein pressures of these rats were measured and cirrhotic liver specimens were obtained. Then, 400 µg of the VEGF/EGFP plasmid was infused through the portal veins of rats in the treatment group, while an equal amount of normal saline was given through the portal vein to rats in the blank group. After 2 wk, portal vein pressures were measured again; then, all rats were sacrificed and liver samples were collected. Comparisons of portal vein pressure were made between the normal and cirrhotic, treated and untreated rats. A randomized controlled trial was carried out to compare the treatment group and the blank group.

Transmission electron microscopy

Fresh specimens were first fixed in 3% glutaral, and then fixed in 1% glutaral after being washed with PBS 3 times for 60 min in total. The samples were dehydrated with an alcohol gradient, embedded in EPON812 epoxy resin, and then cut into 50-nm sections with an Ultrathin microtome. After being dyed with uranyl acetate and lead citrate for 30 min, the sections were observed under transmission electronic microscope (JEM-1200EX, Japan). Ten hepatic sinusoids with a diameter of 2–3 µm were randomly

selected, and the average number of fenestrae per hepatic sinusoid in each state and group was determined. Valid fenestrae ran through LSECs.

Gene array

Angiogenesis microarrays were obtained from SuperArray Bio.Co. (Catalog No. ORN-024). Fresh specimens were put into 4°C of RNA later and incubated for one night. The experiment was performed according to the Oligo GEArray assay protocol. Briefly, total RNA was extracted from tissue using TRIzol reagent (Invitrogen Life Technologies, USA). The quantity and purity of RNA were estimated by measuring A_{260} and A_{280} . A total of 3 µg of RNA was used to synthesize cDNA. The cRNA was then labeled by Biotin-16-dUTP (Roche Cat. No. 1-093-070) and amplified using a TrueLabeling-AMPTM linear RNA amplification kit. The membranes were hybridized with denatured cDNA probe and processed for chemiluminescent detection on X-ray film, and images were acquired using a flatbed desktop scanner. Subsequently, images of spots were converted into numerical data using the free ScanAnalyze software and the raw data was saved as a Microsoft Excel file. All raw signal intensities were corrected for background signal by subtracting the minimum value to avoid the appearance of negative numbers, and were also normalized to the level of a housekeeping gene. These corrected, normalized signals were used to estimate the relative abundance of particular transcripts.

Statistical analysis

Results are expressed as mean \pm SD, and statistical analysis was carried out using the Student's *t*-test and Spearman's rank correlation coefficient for paired data. A *P* value of less than 0.05 was considered to be significant.

RESULTS

VEGF expression *in vitro* and *in vivo*

Twenty-four hours after transfection of cultured hepatocytes with the EGFP/VEGF plasmid, green fluorescent protein was observed in the cytoplasm of liver cells under immunofluorescence microscopy, revealing expression of the plasmid (Figure 1). At the same time, staining of hepatocytes with polyclonal antibodies against VEGF illustrated that these cells expressed immunodetectable VEGF (Figure 2). Moreover, the transfection of liver cells following injection of the EGFP/VEGF plasmid into the portal veins of normal rats *in vivo* was also successful, because a considerable amount of VEGF was identified in the study group, but little was seen in the control group (Figure 3). VEGF was mainly observed in the cytoplasm of hepatocytes and some endothelial cells. The stain integral scores of the study group and control group were 6.0 ± 1.63 and 3.7 ± 2.31 respectively, allowing these groups to be differentiated from each other distinctively (Student's *t* test, $t = 2.74 > 2.62$, $P < 0.05$) (Figure 3).

Fenestrae and portal vein pressures

A comparison was carried out between normal, cirrhotic

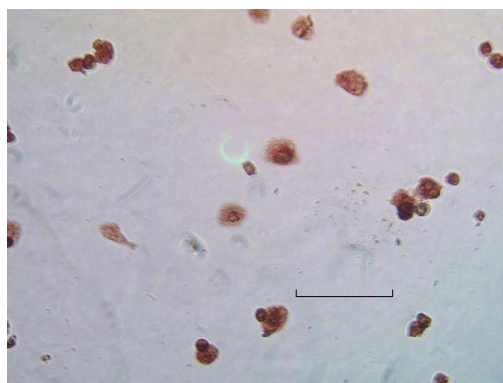


Figure 2 Immunohistochemistry: Positively stained hepatocytes for EGFP/VEGF. Bar denotes 30 μ m.

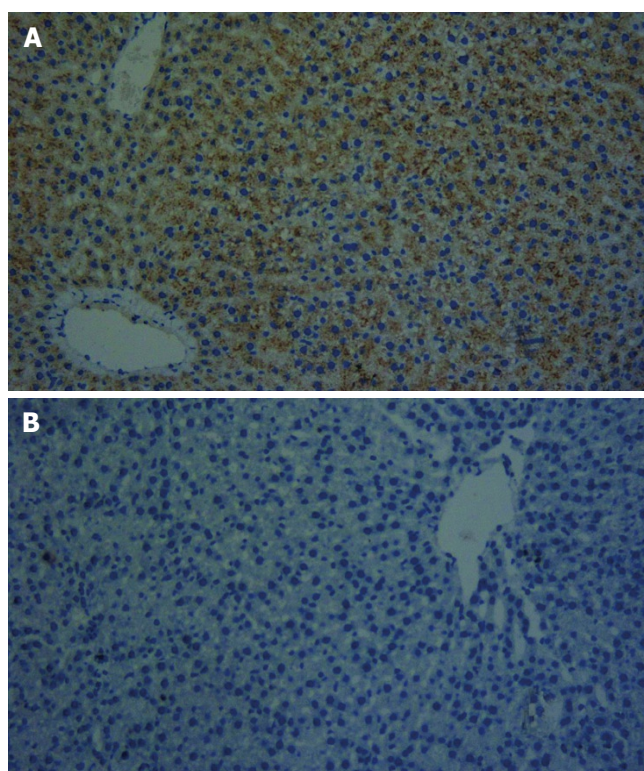


Figure 3 Immunohistochemistry $\times 200$. **A:** Study group injected with EGFP/VEGF plasmid, VEGF-positive hepatocytes $\times 200$; **B:** Control group injected with NS, VEGF-negative hepatocytes.

and treated rats. The number of fenestrae per LSEC in cirrhotic and normal rats was 2.30 ± 1.16 and 7.40 ± 1.71 , respectively (Table 1). The decrease in cirrhotic rats was significant (Student's *t*-test, $t = -7.965 < -6.548$, $P < 0.01$). The portal vein pressures were 17.92 ± 0.90 in cirrhotic rats and 9.32 ± 0.85 in normal rats (Table 1), revealing an obvious difference (Student's *t*-test, $t = 27.32 > 9.30$, $P < 0.01$).

After transfection with the EGFP/VEGF plasmid, the number of fenestrae in the treated group was 4.60 ± 1.65 (Table 1), representing a significant increase compared with cirrhotic rats (Student's *t*-test, $t = -4.14 < -3.56$, $P < 0.05$). The portal vein pressure in the treated group was 15.52 ± 0.93 (Table 1), representing a significant

Table 1 The number of fenestrae and portal vein pressures in different groups ($n = 11$)

Group	The number of fenestrae	Portal vein pressures (mean \pm SD) (cmH ₂ O)
Normal state	7.40 ± 1.71^d	9.32 ± 0.85^d
Cirrhotic state	2.30 ± 1.16	17.92 ± 0.90
Treated group	4.60 ± 1.65^a	15.52 ± 0.93^a
Blank group	2.10 ± 1.10	17.26 ± 1.80

Paired samples *t* test. Significant differences are shown both in fenestrae and pressure. ^a $P < 0.05$ vs Cirrhotic state; ^d $P < 0.01$ vs Blank group.

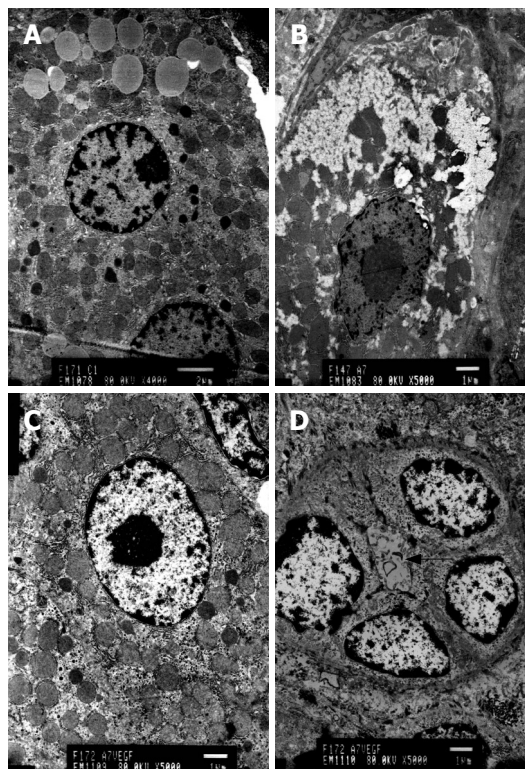


Figure 4 TEM. **A:** Normal hepatocytes, Bar denotes 2 μ m; **B:** Cirrhotic state, hepatocyte apoptosis, Bar denotes 1 μ m; **C:** Cirrhotic rats injected with the EGFP/VEGF plasmid, hepatocyte apoptosis decreased, Bar denotes 1 μ m; **D:** Cirrhotic rats injected with EGFP/VEGF plasmid, Regenerated hepatocytes and cholangiole arrowhead, Bar denotes 1 μ m.

decrease compared with cirrhotic rats (Student's *t*-test, $t = 6.04 > 3.29$, $P < 0.05$).

A randomized controlled trial was performed to compare the treated group and the blank group. There was an obvious difference in the number of fenestrae (4.60 ± 1.65 vs 2.10 ± 1.10 , Student's *t*-test, $t = 4.04 > 3.90$, $P < 0.05$) and in portal vein pressure (15.52 ± 0.93 vs 17.26 ± 1.80 , Student's *t*-test, $t = -3.97 < -3.46$, $P < 0.05$), but there was no significant difference between the cirrhotic group and the blank group in these measures.

Ultrastructural changes in cirrhotic rats after EGFP/VEGF plasmid transfection

Transmission electron microscopy revealed hepatocellular apoptosis in rats with TAA-induced cirrhosis (Figure 4B); moreover, the fenestrae of endothelial cells disappeared

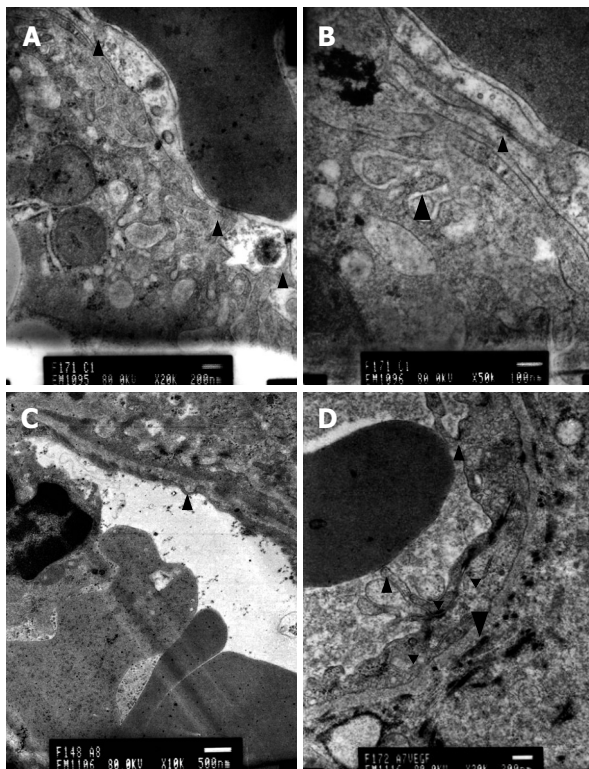


Figure 5 TEM. **A:** Normal state, LSECs and fenestrae (arrowhead). Bar denotes 200 nm; **B:** Normal state. Cell conjunction between LECs (small arrowhead) and microvilli of hepatocytes (large arrowhead). Bar denotes 100 nm; **C:** Cirrhotic state. Fenestrae, cell conjunction and microvilli of hepatocytes disappeared, and a basement membrane appeared (arrowhead). Bar denotes 500 nm; **D:** VEGF treated group. Fenestrae (small arrowhead), microvilli (large arrowhead) and cell conjunctions between LECs (triangle) appeared, Bar denotes 200 nm.

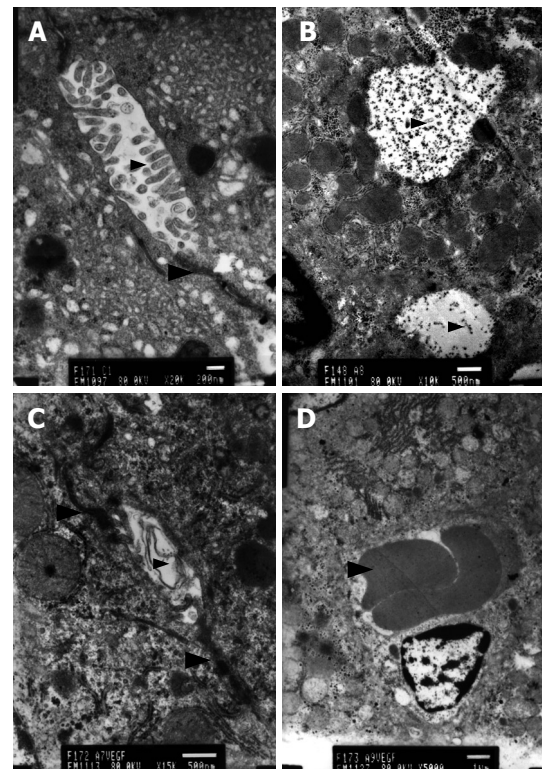


Figure 6 TEM. **A:** Normal state. Cholangiole, microvilli (small arrowhead) and cell conjunction between hepatocytes (large arrowhead). Bar denotes 200 nm; **B:** Cirrhotic state. Microvilli, cell conjunction disappeared and particles of bilirubin (arrowhead) overflowed. Bar denotes 500 nm; **C:** Treated group. Microvilli (small arrowhead) and cell conjunctions (large arrowhead) appeared and bilirubin overflow diminished. Bar denotes 500 nm; **D:** Treated group. Newborn capillary (arrowhead). Bar denotes 1 μ m.

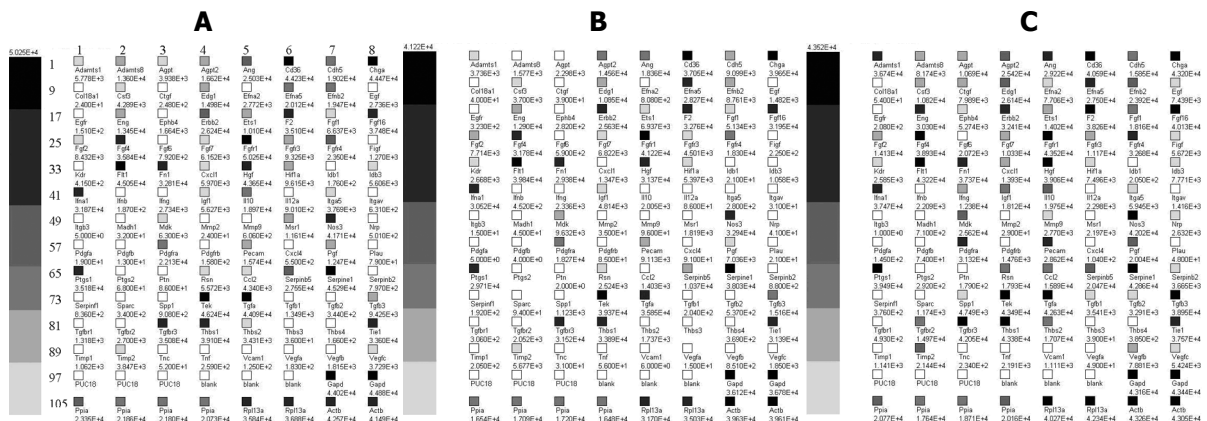


Figure 7 Representative expression profile of angiogenesis-related genes in the liver. The gene microarray included angiogenesis-related genes (1-96), negative control genes (97-99), blank controls (100-102), positive control genes (glyceraldehyde-3-phosphate dehydrogenase (103, 104), cyclophilin A (105-108), ribosomal protein L13a (109, 110) and b-actin (111,112). **A:** Normal state; **B:** Cirrhotic state; **C:** VEGF treated group.

and a basement membrane appeared (Figure 5C). Cell conjunction between hepatocytes was destroyed and particles of bilirubin overflowed into the cytoplasm of hepatocytes and LECs, even into the hepatic sinusoid (Figure 6B). The microvilli of hepatocytes in the space of Disse and the cholangiole were ablated in cirrhotic rats (Figure 5C and Figure 6B). However, this morphology changed after transfection with the EGFP/VEGF plasmid. The fenestrae, cell conjunctions, and microvilli

of hepatocytes were restored, the basement membrane disappeared and cell apoptosis decreased. Newborn capillaries formed by a single liver endothelial cell emerged (Figure 6D).

Gene array

A total of 96 genes involved in angiogenesis were identified on the microarray (Figure 7 and Table 2). The levels of 32 genes were decreased by more than 50% in

Table 2 Angiogenesis-related genes on the angiogenesis microarray

Position	Description	Gene Name
1	A disintegrin-like and metalloprotease (reprolysin type) with thrombospondin type 1 motif, 1	Adamts1
2	Mus musculus a disintegrin-like and metalloprotease (reprolysin type) with thrombospondin type 1 motif	Adamts8
3	Angiopoietin	Angiopoietin 1
4	Mus musculus angiopoietin 2 (Agpt2)	Angiopoietin2
5	Angiogenin	ANG
6	CD36 antigen	CD36
7	Cadherin 5	Cadherin 5
8	Chromogranin A	Chromogranin A
9	Procollagen, type XVIII, alpha 1	COL18A1
10	Colony stimulating factor 3 (granulocyte)	G-CSF
11	Connective tissue growth factor	Fisp12
12	Endothelial differentiation sphingolipid G-protein-coupled receptor 1	Edg1
13	Mus musculus ephrin A2 (Efna2)	Ephrin A2
14	Ephrin A5	Ephrin A receptor
15	Ephrin B2	Ephrin B2
16	Epidermal growth factor	EGF
17	Epidermal growth factor receptor	EGFR
18	Endoglin	Endoglin
19	Eph receptor B4	Ephrin B4
20	V-erb-b2 erythroblastic leukemia viral oncogene homolog 2, neuro/glioblastoma derived oncogene homolog (avian)	Neu/HER2
21	E26 avian leukemia oncogene 1, 5' domain	Ets-1
22	Mus musculus coagulation factor II (F2)	Prothrombin kringle-1
23	Fibroblast growth factor 1	aFGF
24	Fibroblast growth factor 16	FGF16
25	Fibroblast growth factor 2	bFGF
26	Fibroblast growth factor 4	FGF4
27	Fibroblast growth factor 6	FGF6
28	Fibroblast growth factor 7	FGF7/KGF
29	Fibroblast growth factor receptor 1	FLG
30	Fibroblast growth factor receptor 3	FGFR3
31	Fibroblast growth factor receptor 4	FGFR4
32	C-fos induced growth factor	VEGF-D/FIGF
33	Kinase insert domain protein receptor	VEGFR2/FLK 1
34	FMS-like tyrosine kinase 1	VEGFR
35	Mouse fibronectin (FN) mRNA	Fn1
36	Chemokine (C-X-C motif) ligand 1	Gro1
37	Hepatocyte growth factor	HGF
38	Hypoxia inducible factor 1, alpha subunit	Hif1a
39	Inhibitor of DNA binding 1	ID1
40	Inhibitor of DNA binding 3	ID3
41	Interferon alpha family, gene 1	IFNA1
42	Interferon beta, fibroblast	IFN-b1
43	Interferon gamma	IFN r
44	Insulin-like growth factor 1	IGF-1
45	Interleukin 10	IL-10
46	Interleukin 12A	IL-12A
47	Integrin alpha 5 (fibronectin receptor alpha)	Integrin a5
48	Integrin alpha V	CD51
49	Integrin beta 3	CD61
50	MAD homolog 1 (Drosophila)	Smad1
51	Midkine	Midkine
52	Matrix metalloproteinase 2	Gelatinase A
53	Matrix metalloproteinase 9	Gelatinase B
54	Macrophage scavenger receptor 1	SR-A
55	Nitric oxide synthase 3, endothelial cell	NOS3
56	Neuropilin	Neuropilin
57	Platelet derived growth factor, alpha	PDGF a
58	Platelet derived growth factor, B polypeptide	PDGF b
59	Platelet derived growth factor receptor, alpha polypeptide	PDGFRa
60	Platelet derived growth factor receptor, beta polypeptide	PDGFRb
61	Platelet/endothelial cell adhesion molecule	PECAM1
62	Chemokine (C-X-C motif) ligand 4	PF4
63	Placental growth factor	Placental growth factor
64	Plasminogen activator, urokinase	PLAU
65	Prostaglandin-endoperoxide synthase 1	PTGS1
66	Prostaglandin-endoperoxide synthase 2	Cox-2

67	Pleiotrophin	PTN
68	Restin (Reed-Steinberg cell-expressed intermediate filament-associated protein)	Restin
69	Chemokine (C-C motif) ligand 2	Scya2
70	Serine (or cysteine) proteinase inhibitor, clade B, member 5	Maspin
71	Serine (or cysteine) proteinase inhibitor, clade E, member 1	PAI-1
72	Serine (or cysteine) proteinase inhibitor, clade B, member 2	PAI-2
73	Serine (or cysteine) proteinase inhibitor, clade F, member 1	Pedf
74	Secreted acidic cysteine rich glycoprotein	BM-40
75	Secreted phosphoprotein 1	Osteopontin
76	Endothelial-specific receptor tyrosine kinase	Tie-2
77	Transforming growth factor alpha	TGF-a
78	Transforming growth factor, beta 1	TGFb1
79	Transforming growth factor, beta 2	TGF b2
80	Transforming growth factor, beta 3	TGF b3
81	Transforming growth factor, beta receptor I	ALK-5
82	Transforming growth factor, beta receptor II	TGFbR2
83	Transforming growth factor, beta receptor III	Betaglycan
84	Thrombospondin 1	THBS1
85	Thrombospondin 2	THBS2
86	Mus musculus thrombospondin 3 (Thbs-3)	THBS3
87	Mus musculus thrombospondin-4 mRNA	THBS4
88	Tyrosine kinase receptor 1	Tie1
89	Tissue inhibitor of metalloproteinase 1	Timp
90	Tissue inhibitor of metalloproteinase 2	TIMP2
91	Tenascin C	Tenascin C
92	Tumor necrosis factor	TNFa
93	Vascular cell adhesion molecule 1	VCAM-1
94	Vascular endothelial growth factor A	VEGF/VEGI
95	Vascular endothelial growth factor B	VEGF-B
96	Vascular endothelial growth factor C	VEGF-C

Table 3 Relative abundance of transcripts of VEGF and receptor family members

Gene	Normal state	Cirrhotic state	Treated group	Cirrhotic/normal	Treated/cirrhotic
VEGF-D/FIGF	5.790E-2	1.337E-2	2.936E-1	0.2309	21.950
VEGF-C	1.700E-1	1.099E-1	2.807E-1	0.6467	2.5530
VEGF-B	8.275E-2	5.057E-2	4.079E-1	0.6111	8.0650
VEGF/VEGI	8.344E-3	8.914E-4	2.536E-3	0.1068	2.8450
VEGFR	2.054E+0	2.368E+0	2.237E+0	1.1530	0.9448
VEGFR2/FLK1	1.892E-2	1.586E-1	1.338E-1	8.3800	0.8438

cirrhotic rats, and only 8 genes were enhanced by two-fold or greater compared with normal rats. After EGFP/VEGF transfection, 56 genes were increased by two-fold or greater in treated rats; and only one gene was decreased by more than 50% in comparison with cirrhotic rats. In cirrhotic rats, the expression levels of members of the VEGF family were decreased, and the expression levels of members of the VEGF receptor family were increased compared with the levels in normal liver. However, after administration of VEGF, expression of VEGF family members increased and that of receptor family members decreased. Furthermore, the level of VEGF-D was increased by 22-fold, while levels of VEGF, VEGF-B and VEGF-C were increased by more than 2-fold after EGFP/VEGF transfection (Table 3).

DISCUSSION

VEGF-D was first cloned by Yamada in 1997 from a human lung cDNA library^[27]. VEGF-D binds VEGF receptor 2 (VEGF R2/Flk-1/KDR) and VEGF R3 (Flt-4)^[28]. VEGF R2 and VEGF R3 are localized to

vascular and lymphatic endothelial cells and are involved in signaling mediating angiogenesis and lymphangiogenesis^[29]. There are numerous techniques and approaches that have been investigated for gene transfer to the liver. For gene therapy of hepatic diseases in animal experiments, exogenous genes were usually delivered to the liver through the portal vein and bile duct^[30-33]. In our studies, EGFP/VEGF plasmid injection through portal vein was found to be an effective method.

The EGFP/VEGF plasmid was specifically designed for mammalian expression, and was constructed using a human VEGF-D cDNA and pEGFP-N1 plasmid. In the first stage of our studies, we tried to ascertain its expression efficiency, both *in vitro* and *in vivo*. *In vitro*, enhanced green fluorescent protein was observed in the cytoplasm of liver cells under immunofluorescence microscopy. At the same time the staining of hepatocytes with polyclonal antibodies against VEGF illustrated that the cells expressed immunodetectable VEGF. *In vivo*, transfection resulted in a comparatively high expression of VEGF protein. These findings demonstrate the efficient transfection of liver cells with the EGFP/VEGF plasmid *in vitro* and *in vivo*.

VEGF is an important regulator of angiogenesis and vascular permeability, whose expression in the adult is correlated with low permeability of the blood-brain barrier endothelium and high permeability of the fenestrated glomerular endothelium^[14,34]. Fenestrae are critical for the maintenance of the high hydraulic conductivity of the glomerular capillary wall, and their loss results in a reduction in the glomerular filtration rate^[35]. Defenestration is an early event in the pathogenesis of cirrhosis, and precedes the initiation of fibrosis. Sinusoidal capillarization is believed to be important in the initiation of perisinusoidal fibrosis by altering retinol metabolism^[7]. In rats with TAA-induced cirrhosis, the fenestrae of endothelial cell disappeared and a basement membrane appeared. As a result, the microvilli of hepatocytes in the space of Disse were ablated, indicating that material exchange between hepatocytes and hepatic sinusoidal blood failed. Hepatocytes could not obtain the necessary nutrition and eliminate metabolites, which resulted in cell apoptosis. Cell conjunction between hepatocytes was damaged and particles of bilirubin overflowed into the cytoplasm of hepatocytes and endothelial cells, and even into the hepatic sinusoid, resulting in jaundice.

VEGF can promote fenestration and permeability of liver sinusoidal endothelial cells; therefore, it can improve the exchange between hepatic sinusoidal blood and hepatocytes, which argues for the development of VEGF gene therapy for cirrhosis. Roberts and Palade^[36,37] showed that topical administration of VEGF-165 induced fenestrations in continuous microvascular endothelia of muscle and skin, and that tumor neovasculature induced by VEGF is fenestrated. Epithelial cells stably transfected with VEGF cDNA secreted a high level of VEGF and induced a seven- or eight-fold increase in the number of fenestrae and fused clustered vesicles in co-cultured endothelial cells, thus providing direct evidence of a role for VEGF in fenestrae induction. Endothelial cells pretreated with VEGF developed fenestrae and showed increased endothelial permeability^[10]. However, to date, there have been few reports on using VEGF to heal cirrhosis *in vivo*. In our study, after injection of EGFP/VEGF plasmid into cirrhotic rats through the portal vein, the number of fenestrae increased obviously, accompanied with a decrease in portal vein pressure. Hepatocytes and microvillus regeneration demonstrated that the material exchange between hepatocytes and hepatic sinusoidal blood recovered. Furthermore, cell conjunction of hepatocytes was restored and overflow of particles of bilirubin lessened. Meanwhile, the basement membrane disappeared and cell apoptosis decreased. We suspect that the fenestrae and proliferation of LECs played important roles in this change.

At present, there are three points of view on the use of VEGF in cirrhotic disease. The first is that there is decreased VEGF in cirrhosis, and that administration of exogenous VEGF would have a helpful effect^[18,19]. The second is that there is increased VEGF in cirrhosis, and that this VEGF has a harmful effect^[16,17]. The last point of view is that the increased VEGF in cirrhosis is compensative, having a helpful effect^[38]. Our angiogenic

gene array supports the first point of view. We can see clearly that in rats with TAA-induced cirrhosis, the levels of VEGF family members decreased and the levels of VEGF receptors increased, as a reflective compensation. After VEGF/EGFP transfection, the abundance of VEGF family members increased, and that of VEGF receptors decreased, explaining the newborn capillaries we observed by transmission electron microscopy. In our previous study, the density of capillaries was also increased after VEGF-D plasmid hepatic situ injection^[15].

Thus, the levels of transcripts of VEGF family members decreased in TAA-induced cirrhotic livers. Administration of a plasmid encoding an EGFP-VEGF fusion protein attenuated sinusoidal capillarization through increasing the number of fenestrae and the permeability of LECs, which improved the exchange between hepatocytes and sinusoidal blood. Consequently, hepatocytes had more nutrition and oxygen and thus preserved liver function to some extent. Therefore, VEGF gene transfer by injection through the portal vein might be an ideal method for the treatment of cirrhosis.

COMMENTS

Background

Hepatic sinusoidal capillarization has been thought to be a major contributor to hepatic failure in cirrhosis. It includes a defenestrated sinusoidal endothelium and the presence of a subendothelial basement membrane. However vascular endothelial growth factor (VEGF) has been proven to increase the number of fenestrae and permeability of liver sinusoidal endothelial cells (LECs).

Research frontiers

This study was designed to investigate the effect of VEGF transfection on hepatic sinusoidal capillarization by observing the ultrastructural change of cirrhotic endothelium as well as the relationship between the number of fenestrae and portal vein pressure.

Innovations and breakthroughs

Our study shows effective endothelial growth factor (EGFP)/VEGF transfection via injection of plasmid DNA into the portal vein decreases portal pressure in experimental cirrhosis induced by thioacetamide. This effect is mediated by an increase in the formation of fenestrae of liver sinusoids. Previous studies on the role of VEGF in portal hypertension were performed in models of non-cirrhotic pre-hepatic portal hypertension obtained through part portal vein ligation, and showed remarkable angiogenesis effect. The ultra-microstructure in the liver of those rats has not been specifically investigated. This study is more related to what happens in cirrhotic patients.

Applications

Portal hypertension is an almost unavoidable complication of cirrhosis, and it is responsible for the more lethal complications of this syndrome. Previous research has shown the role of hepatic sinusoidal capillarization in cirrhosis. To understand the mechanisms underlying hepatic sinusoidal capillarization and reverse them will be of benefit to developing a new therapeutic method.

Peer review

This paper describes the effect of VEGF transfection on hepatic sinusoidal capillarization in cirrhotic animals as compared to normal rats. The authors have shown histological evidence of partial reversal of cirrhosis.

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BASIC RESEARCH

Effects of α -adrenoreceptor antagonists on apoptosis and proliferation of pancreatic cancer cells *in vitro*

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Abstract

AIM: To discuss the expression of α -adrenoreceptors in pancreatic cancer cell lines PC-2 and PC-3 and the effects of α_1 - and α_2 -adrenoreceptor antagonists, yohimbine and urapidil hydrochloride, on the cell lines *in vitro*.

METHODS: We cultured the human ductal pancreatic adenocarcinoma cell lines PC-2 and PC-3 and analyzed the mRNA expression of α_1 - and α_2 -adrenergic receptors by reverse transcription polymerase chain reaction (RT-PCR). The effects of yohimbine and urapidil hydrochloride on cell proliferation were assessed by 3-(4,5-dimethylthiazol-2-yl)-2,4-diphenyltetrazolium bromide (MTT) assay. Apoptosis was detected using the terminal deoxynucleotidyl transferase (TdT)-mediated biotin-16-dUTP nick-end labeling (TUNEL) assay and flow cytometry (FCM).

RESULTS: PC-2 expressed mRNA in α_1 - and α_2 -adrenoreceptors. MTT assays showed that urapidil hydrochloride had no effect on PC-3 cell lines. However, exposure to urapidil hydrochloride increased DNA synthesis in PC-2 cell lines as compared to the control group. PC-2 cell lines were sensitive to both drugs. The proliferation of the 2 cell lines was inhibited by yohimbine. Cell proliferation was inhibited by yohimbine *via* apoptosis induction.

CONCLUSION: The expression of α_1 - and α_2 -adrenoreceptors is different in PC-2 and PC-3 cell lines, which might be indicative of their different functions. The α_2 -adrenoceptor antagonist, yohimbine, can inhibit the

proliferation of both cell lines and induce their apoptosis, suggesting that yohimbine can be used as an anticancer drug for apoptosis of PC-2 and PC-3 cells.

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Key words: Alpha-adrenoreceptor; Pancreatic cancer; Yohimbine; Urapidil hydrochloride

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INTRODUCTION

Pancreatic cancer is the fourth most common malignancy in the United States. The annual incidence rate is almost identical to the mortality rate, and the 5-year survival rate remains less than 5%^[1]. Approximately 10%-20% of patients have a resectable pancreatic cancer at the time of its diagnosis, and the curative resection rate is only 14% with a median survival of 15-19 mo^[2]. Because of the inability of early diagnosis, early vascular dissemination, and regional lymph node metastasis, pancreatic cancer has a poor prognosis^[3,4].

Recent studies have demonstrated that age, cigarette smoking, alcohol, and chronic pancreatitis are the factors for pancreatic cancer^[5,6]. However, the mechanism of pancreatic cancer is not entirely clear. It was reported that β -adrenoreceptor expressed in cancer of the pancreas, breast, colon, lung, and prostate cell lines is the key factor that induces tumor formation due to long-term smoking and chronic stress^[7-11]. Chronic stress could elevate the level of norepinephrine and epinephrine, leading to β -adrenoreceptor activation. Adrenoceptors modulate diverse intracellular processes, such as DNA synthesis through activation of mitogen-activating protein kinases (MAPKs)^[12].

The α -adrenoceptor subtypes have been classified as α_{1A} -, α_{1B} -, α_{1D} -, α_{2A} -, α_{2B} -, and α_{2C} -adrenoceptors and β -adrenoceptors as β_1 -, β_2 -, and β_3 -adrenoceptors^[13-16]. Various cancer cell lines could express different adrenoceptor subtypes impacting tumor cell biological behavior and heteromorphism. It was recently reported that α_2 -adrenoceptor subtype is expressed in breast cancer cell lines at RNA and protein level. Moreover, these functional receptors are associated with an increase in cell proliferation^[17,18].

In this paper, we discuss the expression of α -adrenoreceptors in pancreatic cancer cell lines PC-2 and PC-3, and the effects of the α_1 - and α_2 -adrenoreceptor antagonists, yohimbine and urapidil hydrochloride *in vitro*. We hypothesize that the α -adrenoreceptor antagonists could suppress the proliferation of pancreatic cancer cells *via* induction of apoptosis. The aim of this study was to describe and characterize the presence of α_1 - and α_2 -adrenoceptors in pancreatic cancer cell lines PC-2 and PC-3 at RNA level using reverse transcription polymerase chain reaction (RT-PCR). Moreover, apoptosis was detected by terminal deoxynucleotidyl transferase (TdT)-mediated biotin-16-dUTP nick-end labeling (TUNEL) assay and flow cytometry (FCM), when pancreatic cancer cells were stimulated with α -adrenoreceptor antagonists.

MATERIALS AND METHODS

Materials

Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco (Grand Island, NY, USA). Trizol was purchased from Invitrogen (Carlsbad, CA, USA). Urapidil hydrochloride, yohimbine, streptomycin, penicillin, and 3,3-dimethyl-2-thionotetrahydro-1,3,5-thiadiazine (MTT) were purchased from Sigma Chemicals (St. Louis, MO, USA).

Cell culture

PC-2 and PC-3 were obtained from the Pathology Department of China Beijing Union Medical University (PC-3 cell line established from human primary exocrine pancreatic head-cancer, PC-2 cell line from human exocrine pancreatic cancer metastasis). Cells were cultured in a high glucose-DMEM medium supplemented with 10% heat-inactivated fetal calf serum, penicillin (100 U/mL), and streptomycin (100 mg/mL) at 37°C in an atmosphere containing 50 mL/L CO₂ and 950 mL/L with a relative humidity.

RT-PCR

RNA was isolated from PC-2 and PC-3 cells lysed with Trizol. Dense RNA was quantified by UV spectrophotometry (at 260/280 nm) and stored at -70°C. For reverse transcriptase reaction, 0.5 μ g of 18 oligo (dT) primers in diethyl pyrocarbonate (DEPC) water was heated to 70°C for 5 min to allow annealing of the primer to RNA followed by chilling on ice. A total of 10 mmol/L dNTPs, 20 units of RiboLock ribonuclease inhibitor, 5 \times reaction buffer [250 mmol/L Tris-HCl (pH 8.3), 250 mmol/L KCl, 20 mmol/L MgCl₂, and 50 mmol/L dithiothreitol (DTT)]

were added and the reaction mixture was incubated at 37°C for 5 min. Then, 200 units of M-MuLV reverse transcriptase was added and the mixture was incubated at 42°C for 1 h, followed by heat inactivation for 10 min at 70°C.

PCR was performed with 5 μ L RT reaction mixture containing 1 μ L of 10 mmol/L dNTPs, 5 μ L of 10 \times PCR buffer [100 mmol/L Tris-HCl (pH 8.3), 500 mmol/L KCl, and 15 mmol/L MgCl₂, 1.5 μ L of Taq polymerase (Perkin Elmer, Branchburg, NJ, USA), 1 μ L primers for human α_{1A} -, α_{1B} -, α_{1D} -, α_{2A} -, α_{2C} -, α_{2B} -, β_1 -, and β_2 -adrenoceptors, and DEPC water in a final volume of 50 μ L. Adrenoceptor primers were designed according to Genbank, NCBI. The α_{1A} adrenoceptor primers (sense: 5'-CTCTGCGTCTGGGCACTCT-3' and antisense: 5'-GAAGCAGCCGACCACGAT-3') were used to amplify a 402-bp fragment. The α_{1B} primers (sense: 5'-AAGAACTTTCACGAGGACACCC-3' and antisense: 5'-CAGAACACCACCTTGAACACG-3') were used to amplify a 233-bp fragment. The α_{1D} primers (sense: 5'-TCACTCAAGTACCCAGCCATCA-3' and antisense: 5'-GGAACCAGCAGAGCACGAAG-3') were used to amplify a 487-bp fragment. The α_{2A} primers (sense: 5'-ATCGGAGTGTTCTGTTG-3' and antisense: 5'-AGGAAGCAATAGTGATTAGGG-3') were used to amplify a 489-bp fragment. The α_{2C} primers (sense: 5'-TGTTCGTGCTCTGCTGGTTC-3' and antisense: 5'-GGGGAAGGCAAAGGGGTC-3') were used to amplify a 427-bp fragment. The α_{2B} primers (sense: 5'-AAATCGGGGCGACAATAG-3' and antisense: 5'-GAGACCCAAGCCACTAAAA-3') were used to amplify a 367-bp fragment. The β_1 primers (sense: 5'-GGGAGAAGCATTAGGG-3' and antisense: 5'-CAAGGAAAGCAAGGTGGG-3') were used to amplify a 270-bp fragment. The β_2 primers (sense: 5'-CAGCAAAGGGACGAGGTG-3' and antisense: 5'-AAGTAATGGCAAAGTAGCG-3') were used to amplify a 334 bp fragment. The PCR conditions for the receptors of the above-mentioned primers were as follows: 1 cycle at 94°C for 2 min; 35 cycles at 94°C for 30 s, at 62°C for 60 s, and at 70°C for 2 min; and a final extension at 70°C for 7 min. Reactions were run on a MJ Research PTC-200 thermal cycler. The PCR products and a 100-bp DNA ladder were run on a 1.5% agarose gel for 2 h at 90 V. The gel was imaged using ethidium bromide staining with a UVP GDS 7500 or an Ultra Lum TUI-5000 gel documentation system.

MTT assay

The effects of yohimbine and urapidil hydrochloride on cell proliferation were detected by colorimetric MTT assay based on the NADH-dependent enzymatic reduction of the tetrazolium salt MTT in metabolically active cells but not in dead cells. Cells were seeded in a basal medium at the concentration of 1×10^4 cells/well in 96-well plates, and allowed to grow and adhere in complete media at 37°C with 50 mL/L CO₂ for 20-24 h. The cells were then transferred to basal media with 0.05% FBS and incubated for 24 h with control (no treatment), yohimbine (25-200 μ mol/L), and urapidil hydrochloride (25-200 μ mol/L). Following incubation, 20 μ L MTT (5 mg/mL) was added to each well, and the cells grew in complete media at 37°C with 50 mL/L CO₂ for 4 h. The supernatant was removed,

then 150 μ L DMSO was added to each well of the 96-well plate and swung for 10 min. The optical density at 490 nm was determined with an enzyme linked immunosorbent assay (ELISA) reader.

TUNEL assay

TUNEL assay was performed with the *in situ* cell apoptosis detection kit following its manufacturer's instructions. Briefly, the cells were replaced on cover slips after exposure to urapidil and yohimbine for 24 h and fixed with 4% paraformaldehyde for 30 min and adhered to slides with balsam. Nonspecific chromogen reaction, induced by endogenous peroxidase, was inhibited with 3% H₂O₂ for 10 min at room temperature. Terminal deoxynucleotidyl transferase (TdT) and biotin-11-dUTP reactions were performed for 1 h at 37°C in a humidified box, and blocking reagent was applied for 30 min at room temperature, followed by Avidin-HRP for 1 h at 37°C in a humidified box. For biochemical controls, positive control slides were treated with DNase and negative control slides were treated with PBS instead of TdT. DNA fragments were stained using DAB as a substrate for peroxidase, and hematoxylin was used as a counter stain. Apoptotic index was calculated as a ratio of the number of apoptotic cells to the total number of tumor cells in each slide.

Analysis of the apoptosis rate by annexin V and FITC/PI FCM

The apoptosis rate was measured by FCM according to the instructions supplied with the annexin V-FITC kit. In brief, after treatment with urapidil and yohimbine, PC-2 and PC-3 cells were harvested by centrifugation, washed once with ice-cold PBS and resuspended in binding buffer at a concentration of 1×10^6 cells/mL, in which 100 mL of cell suspension was added in a 5 mL FCM tube. A total of 5 mL of annexin V-FITC and 10 mL of 20 mg/mL PI were added and incubated for 15 min in the dark prior to a further addition of 400 mL PBS. Quantitative analysis of apoptotic level was performed using a flow cytometer (BD Biosystems, CA, USA). The apoptotic percentage of 10000 cells was determined and all the experiments reported in this study were performed in triplicate.

Statistical analysis

In vitro data were analyzed by one-way ANOVA and nonparametric Kruskal-Wallis test by using SPSS version 13.0. Values were expressed as mean \pm SD, and $P < 0.05$ was considered statistically significant.

RESULTS

RT-PCR

RT-PCR results demonstrated that PC-2 expressed mRNA in α_{1D} , α_{1A} , α_{2A} , β_1 and β_2 -adrenoceptors and PC-3 expressed mRNA in α_{2B} , β_1 , and β_2 -adrenoceptors (Figure 1).

MTT assay

MTT assays showed that urapidil hydrochloride had no effect on PC-3 cell lines, whereas exposure to urapidil hydrochloride increased DNA synthesis in the PC-2 cell line as compared to the control group. PC-2 was sensitive to

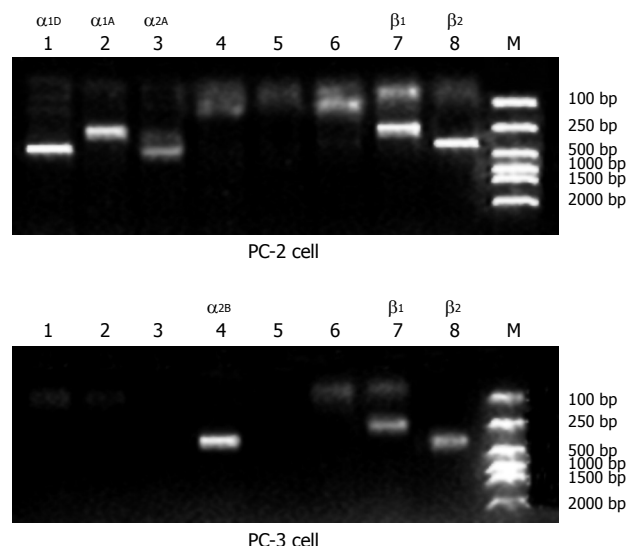


Figure 1 Expression of mRNA in adrenoceptors of human ductal pancreatic adenocarcinoma cell lines PC-2 and PC-3 by RT-PCR. Lanes 1-8 represent the α_{1D} -, α_{1A} -, α_{2A} -, α_{2B} -, α_{2C} -, α_{1B} -, β_1 -, and β_2 -adrenoceptor subtypes, respectively. Lane maker = 100 bp DNA ladder. PC-2 expressed mRNA in α_{1D} -, α_{1A} -, α_{2A} -, β_1 -, and β_2 -adrenoceptors and PC-3 expressed mRNA in α_{2B} -, β_1 -, and β_2 -adrenoceptors.

Table 1 α -adrenoceptor antagonists-induced apoptosis in human pancreatic cancer cells (mean \pm SD)

Treatment	TUNEL-positive cells (apoptotic index) (%)			
	PC-2 100 μ mol/L	PC-2 200 μ mol/L	PC-3 100 μ mol/L	PC-3 200 μ mol/L
Yohimbine	20.91 \pm 2.43 ^a	39.41 \pm 3.21 ^a	15.47 \pm 2.19 ^a	21.22 \pm 2.57 ^a
Urapidil	1.57 \pm 0.94	2.47 \pm 1.03	1.98 \pm 0.69	2.33 \pm 0.92
Control	2.56 \pm 0.78		2.14 \pm 0.62	

^a $P < 0.05$ vs control.

both drugs. The proliferation of 2 cell lines was inhibited by yohimbine. However, the rate of suppression by yohimbine was weaker than that by 5-FU used as a positive control (Figure 2).

TUNEL assay

Nuclei of apoptotic cells were condense and stained brown whereas the negative cells were stained blue in the TUNEL assay. The results were in accordance with the MTT assay and revealed that yohimbine could induce apoptosis of both cell lines whereas urapidil hydrochloride had no effect on the 2 cell lines (Figure 3 and Table 1).

FCM

PC-2 and PC-3 cells were treated with the α -adrenoceptor antagonists, urapidil hydrochloride (200 μ mol/L) and yohimbine (200 μ mol/L) for 24 h. The cells treated with urapidil hydrochloride and yohimbine were double stained with annexin V and PI and analyzed by FCM (Figure 4).

DISCUSSION

In this study, α_1 -adrenoceptors were only expressed in

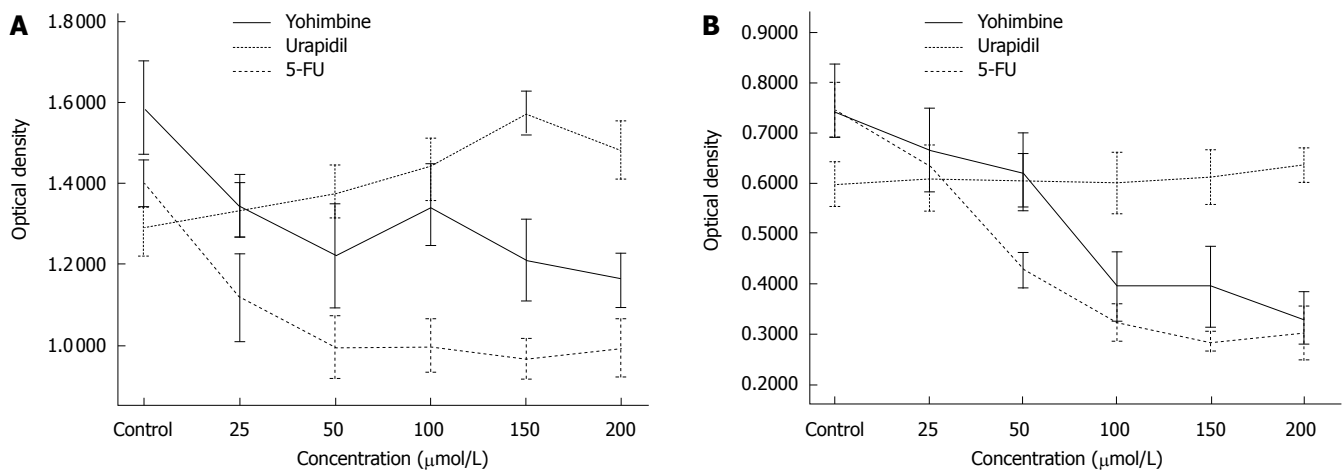


Figure 2 MTT assay shows that PC-2 cells treated with urapidil hydrochloride for 24 h could increase cellular proliferation in a concentration-dependent manner. Yohimbine inhibited the proliferation of PC-2 cells (A) and urapidil hydrochloride had no effect on PC-3 cell lines while the proliferation of PC-3 cell lines was inhibited by yohimbine (B).

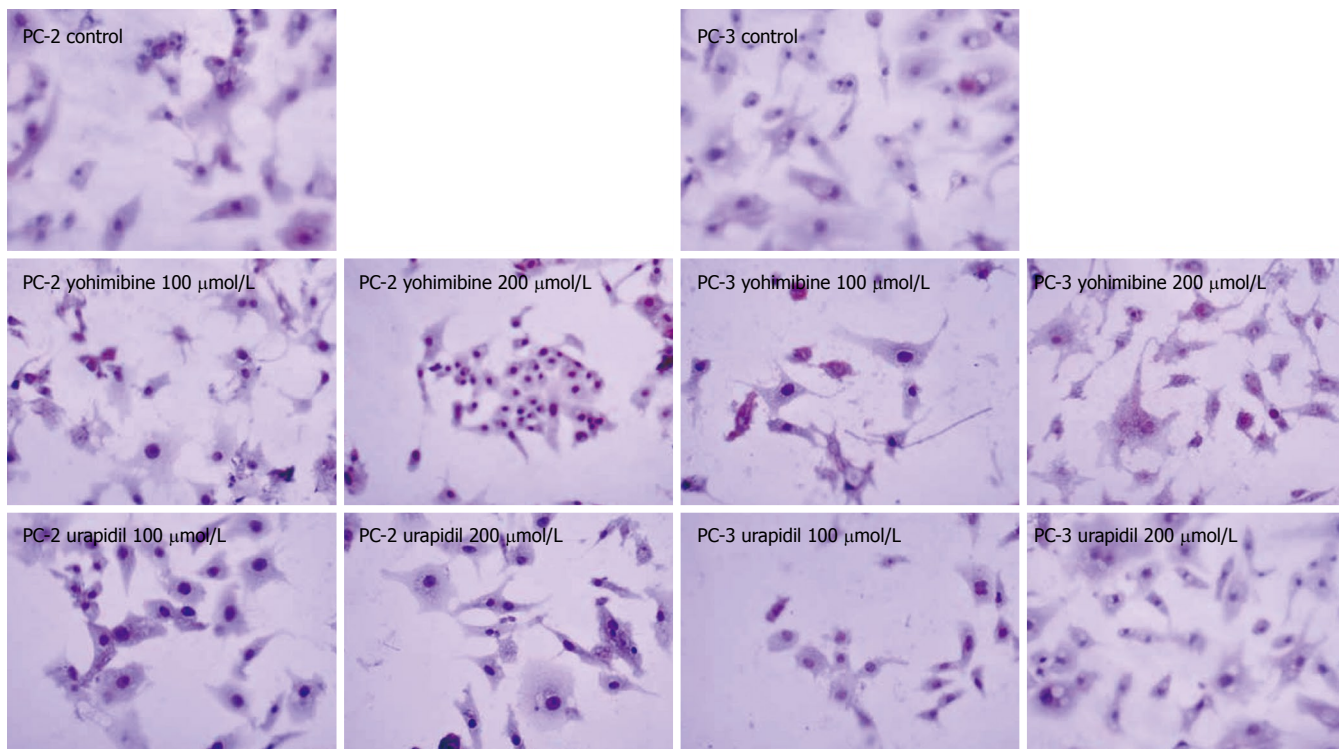


Figure 3 TUNEL assay showing apoptotic PC-2 and PC-3 cells after treatment with 100 μmol/L urapidil hydrochloride and yohimbine for PC-2 cells and 100 μmol/L yohimbine and 100 μmol/L urapidil for PC-3 cells compared with control group.

PC-2 cells, while α_2 -adrenoceptors were expressed in both PC-2 and PC-3 cells. MTT assay demonstrated that urapidil hydrochloride had no effect on PC-3 cell line, but increased DNA synthesis in PC-2 cell line as compared to the control group. The proliferation of the 2 cell lines was inhibited by yohimbine. The rate of suppression by yohimbine was weaker than by 5-FU used as a positive control.

α -adrenoceptor is a member of the superfamily of G protein-coupled adrenoceptors, which mediate reactions of endogenous catecholamines in a variety of target cells^[12]. Currently, the following 6 native α -adrenoceptor subtypes have been identified: α_{1A} , α_{1B} , α_{1D} , α_{2A} , α_{2C} , and

α_{2B} , which have been studied more extensively in diseases of the cardiovascular system, such as vascular constriction and hypertension mechanism^[17,20,21]. However, there are few reports on the relationship between α -adrenoceptor subtypes and malignant tumors. Our study identified the mRNA expression of α -adrenoceptor subtypes in PC-2 and PC-3 cell lines, and showed that the PC-2 cell line expressed α_{1D} -, α_{1A} -, and α_{2A} -adrenoceptor subtype mRNA, and the PC-3 cell line expressed the α_{2B} -adrenoceptor subtype mRNA.

In the present study, prostatic cells expressed α_1 -adrenoceptors. Catecholamine could promote prostatic cell proliferation^[22]. Growth of human prostate cancer

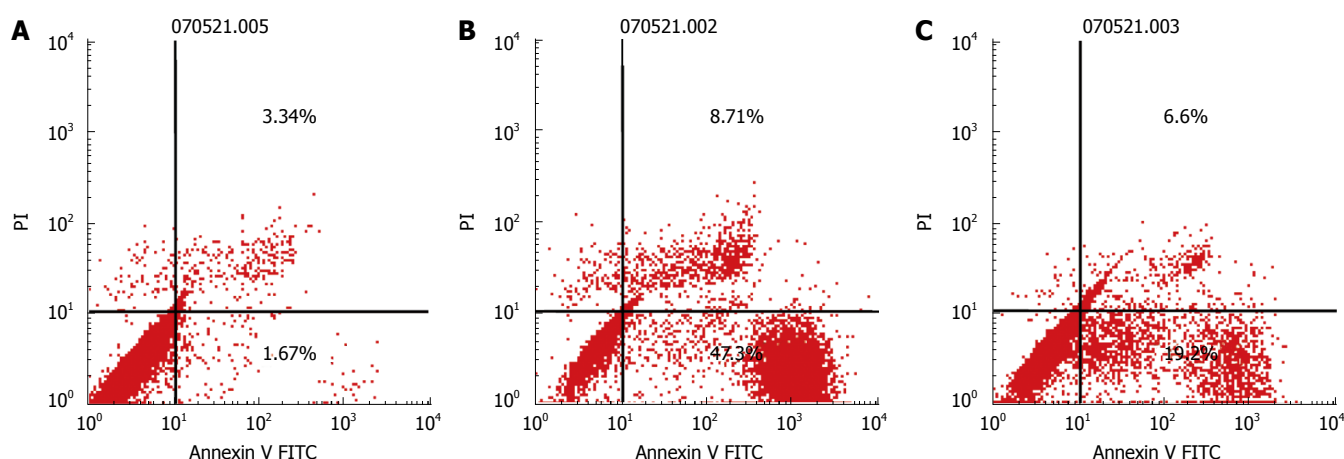


Figure 4 Apoptotic PC-2 and PC-3 cells after treatment with 200 $\mu\text{mol/L}$ urapidil hydrochloride (A) and 200 $\mu\text{mol/L}$ yohimbine (B) for PC-2 cells and 200 $\mu\text{mol/L}$ yohimbine for PC-3 cells (C) detected by FCM. Significant apoptosis was induced by yohimbine on both PC-2 and PC-3 cells, whereas urapidil hydrochloride had no apoptotic effect on PC-2 cell.

cells could be suppressed by α_1 -adrenoceptor antagonists, doxazosin and terazosin, by inducing apoptosis^[23,24]. On the other hand, the α_1 -adrenoceptor antagonist, urapidil hydrochloride, in our study, had effects on the proliferation of PC-2 cells but not on that of PC-3 cells, which might be related to the α -adrenoceptor subtype, but the mechanism of action is not clear.

α_2 -adrenoceptor is classically linked to the inhibition of adenylyl cyclase leading to a reduction in intracellular cAMP. Recent studies revealed that yohimbine inhibits both intracellular and extracellular cAMP levels in human mammary tumor MCF-7 cells and also suppresses the proliferation of MCF-7 cells, indicating that α -adrenergic receptors may mediate this kind of action^[16,19]. Our study demonstrated that the α_2 -adrenoceptor antagonist, yohimbine, could inhibit PC-2 and PC-3 cell proliferation by inducing apoptosis.

The molecular mechanism underlying apoptosis of PC-2 and PC-3 cells induced by yohimbine is unknown. It was reported that expression of a cloned α_2 -adrenergic receptor allows the coupling of this receptor to the p21^{ras}-mitogen-activated protein (MAP) kinase cascade in hamster lung fibroblasts via a Gi-mediated pathway that is independent of adenylyl cyclase inhibition or phospholipase stimulation^[24,25]. MAPK pathway plays a pivotal role in pancreatic cancer progression by stimulating distinct mitotic, antiapoptotic, and angiogenic cascades, and by controlling different intracellular signaling elements activated by other growth factors in pancreatic cancer cells^[26]. Based on our hypothesis that yohimbine induces apoptosis of PC-2 and PC-3 cells by inhibiting the MAPK or other pathways, our group will document the molecular mechanism underlying the apoptosis induced by yohimbine in future studies.

In conclusion, α_2 -adrenoceptor antagonist, yohimbine but not urapidil hydrochloride, exerts a negative effect on pancreatic cancer cell growth by inducing apoptosis. Although the potential underlying mechanism is presently unknown, yohimbine may suppress pancreatic cell growth by deregulating signal transduction pathways, potentially

involving the MAPK or other pathways. The mechanistic aspects of the antitumor effect of yohimbine against pancreatic tumors are under investigation.

COMMENTS

Background

It is supposed that neurobiology would play a key role in development of malignant tumors, by its direct effect on malignant tumors or on tumor micro-environment. Autonomic nerves are closely related with various activities of the body. A few reports are available on the beta-adrenergic receptors in cancer of colon, breast, and prostate, etc. However, little is known about alpha-adrenergic receptors in malignant tumor, we, therefore, studied the distribution and function of alpha-adrenergic receptors of malignant tumor cells.

Research frontiers

Pancreas is a complex organ, including blood, nerve and lymph. Pancreatic cancer is generally characterized by less blood supply, innervation complex, and lymphatic irregularity, which have been brought difficulty to diagnosis and treatment of pancreatic cancer.

Innovations and breakthroughs

In recent years, basic researches of malignant tumor concentrated on their biological characteristics, such as invasion and metastasis. This study explored the development, metastasis and infiltration of pancreatic cancer by neurobiology.

Applications

The study is only a part of our research. The study would screen the drugs suppressing malignant tumor, from cardiovascular drugs.

Peer review

The study investigated the effects of α -adrenoreceptor antagonists on proliferation and apoptosis of pancreatic cancer cells. The subject is important because very little is known about the role of adrenergic stimulation in the regulation of proliferation and death of pancreatic cancer cells. The study was well designed.

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CLINICAL RESEARCH

Efficacy, risk factors and complications of endoscopic polypectomy: Ten year experience at a single center

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Abstract

AIM: To examine the efficacy and complications of colonoscopic resection of colorectal polypoid lesions.

METHODS: We retrospectively reviewed 1354 polypectomies performed on 1038 patients over a ten-year period. One hundred and sixty of these were performed for large polyps, those measuring ≥ 20 mm. Size, shape, location, histology, the technique of polypectomy used, complications, drugs assumption and associated intestinal or extra intestinal diseases were analyzed. For statistical analysis, the Pearson χ^2 test, NPC test and a Binary Logistic Regression were used.

RESULTS: The mean patient age was 65.9 ± 12.4 years, with 671 men and 367 women. The mean size of polyps removed was 9.45 ± 9.56 mm while the size of large polyps was 31.5 ± 10.8 mm. There were 388 pedunculated and 966 sessile polyps and the most common location was the sigmoid colon (41.3%). The most frequent histology was tubular adenoma (55.9%) while for the large polyps was villous (92/160 -57.5%). Coexistent malignancy was observed in 28 polyps (2.1%) and of these, 20 were large polyps. There were 17 procedural bleeding (1.3%) and one perforation. The statistical analysis showed that cancer is correlated to polyp size ($P < 0.0001$); sessile shape ($P < 0.0001$) and bleeding are correlated to cardiac disease ($P = 0.034$), tubular adenoma ($P = 0.016$) and polyp size.

CONCLUSION: The endoscopic resection is a simple and safe procedure for removing colon rectal neoplastic lesions and should be considered the treatment of choice for large colorectal polyps. The polyp size is an important risk factor for malignancy and for bleeding.

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Key words: Colonoscopy; Polypectomy; Large polyps; Colorectal neoplastic lesions; Endoscopic resection

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INTRODUCTION

The colonoscopic polypectomy was introduced by Wolf and Shinya in the early 1970s and has become the most common therapeutic procedure performed within endoscopic units^[1]. Colonoscopic polypectomy is the first approach and the standard treatment for colon rectal polyps and, therefore, a prevention of colorectal cancer. It is a safe technique when performed by expert hands using a cautious technique and equipment that works properly; so that, in these conditions, complications should be an uncommon event.

Some factors can increase the rate of complications such as the type (pedunculated or sessile) and size of the polyp, its location and other factors regarding the comorbidity of the patient (coagulation disorders or drug assumption) and the technique used. The most common complications after polypectomy are bleeding (from 0.3% to 6.1%) and pain due either to excessive gas accumulation or to parietal damage and perforation after current

application^[2]. Frequently, these complications follow large polyps polypectomies. Several authors have reported complications during the endoscopic removal of large polyps and in the majority of these studies, “large” was defined as equal to or greater than 20 mm.

Large polyps represent a particular challenge for the endoscopist because they are often related to important risks of haemorrhage (0%-22.1%), perforation (0%-1.3%) and inadequate polypectomy^[3-19]. The alternative to endoscopic therapy of large polyps is the surgical resection which involves hospitalization and anaesthesia. In addition, the risks of surgery are significant, especially in elderly patients with comorbid diseases. Moreover, the higher mortality (2%-4%) and morbidity (10%) rates reported for surgery with respect to endoscopic polypectomy cannot be overlooked^[3].

This study aims to show a retrospective series of endoscopic resections of colon rectal polyps and in particular of large polyps, focusing on the efficacy, complications and risk factors.

MATERIALS AND METHODS

From January, 1996 to May, 2006, 1038 patients underwent colonoscopy for the removal of 1354 polypoid lesions. Of these polypectomies, 160 (11.8%) were performed on large polyps, those measuring 20 mm or more. The patients were prepared for colonoscopy following standard protocol, following a fiber and residue free diet within 72 h before the investigation and the assumption of 4000 mL of a polyethylene glycol electrolytic lavage solution 18 h before colonoscopy. The colonoscopy was performed according to standard procedure using two Olympus CF-100 HI and one Pentax EC3830FK videocolonoscopes. An electrocautery Erbe ICC 200 and argon plasma coagulation Erbe APC 300 were also used. The sclerosis needle and endoscopic snare used during the polypectomy procedure were the standard models. All procedures were performed by two expert endoscopists.

For the statistical analysis the polyps were split in four groups: the first group represented polyps measuring between 1-10 mm, the second 11-19 mm, the third 20-39 mm and the fourth group of polyps measuring 40 mm or more. For shape, polyps were grouped in pedunculated (pedunculated and semi-pedunculated) and sessile (sessile and flat-elevated). The method of resection was selected according to the size and morphologic features of each lesion and three groups were created: direct snare (hot or cold biopsy and hot or cold snare in smaller lesions and larger protruding lesions), mucosectomy (en bloc, piecemeal, the inject and cut and the inject, lift, and cut for broad-based lesions) and endoloop.

Pedunculated polyps were transected at the stalk just below the polyp head. In all pedunculated polyps injection into the base of the stalk was not performed, before polypectomy, as a prophylactic measure to prevent the bleeding, except for the cases of pedunculated polyps with a very big stalk where bleeding prophylaxis was performed with the application of endoloop. Sessile polyps were resected using two techniques, en bloc and piecemeal.

All resected material was retrieved for histological examination.

Post-polypectomy bleeding was defined as procedural if it occurred during polypectomy, immediate if it occurred within 24 h of polypectomy, and delayed if it occurred more than 24 h after the procedure. Bleeding was treated by injection therapy, with dilute adrenalin at a concentration of 1:10 000 with or without 1% polidocanol, hemoclips and thermal coagulation using argon plasma. The variables evaluated in the study were: polyp size, polyp shape, their location along the colon, complications (bleeding and perforation), histology, technique of polypectomy applied, drugs assumption and associated intestinal or extra intestinal diseases.

Continuous data are described by mean and standard deviation or median and range, according to distribution. Categorical data are presented as numbers and percentages. The Pearson χ^2 test with Brandt-Snedecor and Kimball's formula^[20] was used to assess the association between categorical variables while to individualize the correlation between categorical variables and some numerical variables a biserial correlation coefficient was used^[21]. Differences between the groups were evaluated using the non parametric combination NPC Test, based on permutation tests^[22]. A *P*-value < 0.05 was considered statistically significant. The estimation of a Binary Logistic Regression model allowed for the individualization of the variables which were tied on the bleeding^[23]. In this context, the estimation of Log-Likelihood test and G test allows for the obtainment of the measure of goodness-of-fit.

Software used included SPSS, Windows 11.0 (2001) for Pearson χ^2 test and biserial correlation, Microsoft Excel (2002) for the Brandt-Snedecor method and Kimball's formula, Metodologica S.R.L. (2001) for nonparametric analysis NPC test and Minitab Release 13.31, Copyright © 2000 Minitab Inc. for Binary Logistic Regression.

RESULTS

The demographic and clinical data of the patients studied and the characteristics of polyps according to size are illustrated in Table 1 and Table 2. Among 1354 endoscopic polypectomies, 907 were lesions less than 10 mm of diameter, 287 between 11 and 19 mm, and 160 more than 20 mm; the size of total polyps was 9.45 ± 9.56 (range 1-100) while the size of large polyps was 31.5 ± 10.8 . Macroscopically sessile shapes were prevalent (966/1354 -71.3%) and the pedunculated ones were 388 (28.7%).

The most frequent location of the polyps was the sigmoid colon (559/1354 -41.3%). The most frequent location of large polyps was the sigmoid colon (65/160 -40.7%).

The most commonly used technique was direct endoscopic snare resection in 1294 polypectomies (95.6%) as well as in the group of large polyps 132/160 (82.5%).

The most frequent histological type among all the polyps resected was tubular adenoma, 756/1354 -55.9%), while, for the large polyps, it was the villous type (92/160 -57.5%). Of the removed polyps, 1078 were adenomas with low grade dysplasia (79.6%), 123 with high grade dysplasia (9.1%) and 28 (2.1%) were adenomas containing

Table 1 Demographic and clinical data of the populations studied

	Total	Group 1	Group 2	Group 3	Group 4
Number of patients	1038	675	211	121	31
Age	65.9 ± 12.4	65.1 ± 12.9	67.1 ± 11.9	68.1 ± 10.3	71.9 ± 9.9
Sex					
M/F	671/367	448/227	133/78	73/48	17/14
Drugs assumption					
Aspirin or anticoagulant	22 (2.1%)	12 (1.77%)	6 (2.8%)	4 (3.3%)	
Associated extra intestinal diseases					
Cardiac diseases	70 (6.7%)	47 (6.96%)	13 (6.16%)	8 (6.6%)	
Diabetes Mellitus	8 (0.7%)	8 (1.18%)			
Chronic Renal Failure	11 (1.05%)	4 (0.6%)	4 (1.89%)	2 (1.65%)	1 (3.22%)
Neoplasms	32 (3.08%)	18 (2.6%)	8 (3.79%)	5 (4.1%)	1 (3.22%)
Liver diseases	34 (3.3%)	26 (3.85%)	5 (2.36%)	3 (2.47%)	
Endocrinological diseases	1 (0.09%)	1 (0.14%)			
Associated intestinal diseases					
Diverticula	262 (25.2%)	188 (27.8%)	51 (24.1%)	17 (14.04%)	6 (19.3%)
CRC	112 (10.8%)	60 (8.88%)	36 (17.06%)	16 (13.2%)	
IBD	31 (2.9%)	22 (3.25%)	8 (3.79%)	1 (0.8%)	
Ischemic colitis	8 (0.7%)	7 (1.03%)		1 (0.8%)	
Melanosis coli	10 (0.96%)	8 (1.18%)	2 (0.94%)		
Emorroidi	39 (3.75%)	27 (4%)	9 (4.26%)	3 (2.47%)	
Angiodysplasia	13 (1.25%)	12 (1.77%)	1 (0.47%)		

Table 2 Characteristics of polyps resected according to size

	Total	Group 1	Group 2	Group 3	Group 4
Number of polypectomy	1354	907	287	129	31
Size (mm)	9.45 ± 9.56	4.8 ± 2.18	11.8 ± 2.6	27.7 ± 5.8	46.8 ± 13.02
Range	(1-100)	(1-10)	(11-19)	(20-39)	(40-100)
Shape					
Pedunculated	388 (28.7%)	176 (19.4%)	135 (47%)	70 (54.3%)	7 (22.6%)
Sessile	966 (71.3%)	731 (80.6%)	152 (53%)	59 (45.7%)	24 (77.4%)
Location					
Rectal	247 (18.3%)	146 (16.1%)	54 (18.8%)	33 (2.6%)	14 (45.4%)
Sigmoid	559 (41.3%)	375 (41.3%)	119 (41.5%)	59 (45.7%)	6 (19.4%)
Left colon	184 (13.6%)	126 (13.9%)	44 (15.3%)	13 (10.1%)	1 (3.2%)
Splenic flexure	24 (1.8%)	14 (1.6%)	8 (2.8%)	1 (0.8%)	1 (3.2%)
Transverse	94 (6.9%)	69 (7.6%)	17 (5.9%)	6 (4.7%)	2 (6.4%)
Hepatic flexure	64 (4.7%)	45 (4.9%)	12 (4.2%)	3 (2.3%)	4 (12.8%)
Right colon	129 (9.5%)	95 (10.5%)	23 (8%)	9 (6.9%)	2 (6.4%)
Caecum	53 (3.9%)	37 (4.1%)	10 (3.5%)	5 (3.9%)	1 (3.2%)
Histology					
Tubular adenoma	756 (55.9%)	596 (65.8%)	138 (48.1%)	19 (14.7%)	3 (9.7%)
Villous adenoma	243 (17.9%)	103 (11.3%)	48 (16.7%)	66 (51.2%)	26 (83.9%)
Tubulovillous adenoma	315 (23.3%)	174 (19.2%)	97 (33.8%)	42 (32.6%)	2 (6.4%)
Hyperplastic	40 (2.9%)	34 (3.7%)	4 (1.4%)	2 (1.5%)	
Dysplasia					
No	125 (9.2%)	117 (12.9%)	6 (2.1%)	2 (1.5%)	
LGD	1078 (79.6%)	769 (84.8%)	245 (85.4%)	57 (44.2%)	7 (22.6%)
HGD	123 (9.1%)	17 (1.9%)	32 (11.1%)	51 (39.5%)	23 (74.2%)
Invasive cancer	28 (2.1%)	4 (0.4%)	4 (1.4%)	19 (14.8%)	1 (3.2%)
Technique of polypectomy					
Direct snare	1294 (95.6%)	907 (100%)	255 (88.9%)	108 (83.7%)	24 (77.4%)
Mucosectomy	56 (4.1%)		30 (10.4%)	19 (14.8%)	7 (22.6%)
Endoloop	4 (0.3%)		2 (0.7%)	2 (1.5%)	
Complication					
Bleeding	17 (1.3%)	4 (0.4%)	3 (1.05%)	7 (5.4%)	3 (9.7%)
Perforation	1 (0.07%)		1 (0.35%)		
Technique of hemostasis					
Injection	13 (76.5%)	4 (100%)	2 (66.7%)	6 (85.7%)	1 (33.3%)
Clips	3 (17.6%)		1 (33.3%)		2 (66.7%)
APC	1 (5.9%)			1 (14.3%)	

an area of invasive carcinoma and of these 20 were large polyps. The estimation of biserial correlation coefficient

allows us that the cancer and polyp size are correlated ($P < 0.0001$), but there wasn't a significant correlation

Table 3 Results of the Pearson χ^2 test applied in the sub-groups for evaluation of the association between bleeding and variables

	P-value			
	Group 1	Group 2	Group 3	Group 4
Associated extra intestinal diseases				
Liver diseases	0.003	0.060	0.001	0.851
Location				
Transverse colon	0.0001	0.966	0.853	0.123
Histology				
Hyperplastic polyp	0.238	0.023	0.912	0.125
Dysplasia				
Invasive cancer	0.996	0.0001	0.681	0.561
Associated intestinal diseases				
Colon rectal cancer	0.130	0.384	0.922	0.036

between cancer and age ($P = 0.464$). To evaluate the association between malignancy and sex, histology, location and shape, we applied a χ^2 test, where it showed significant results for the association with shape ($P < 0.0001$), in particular sessile and sex ($P < 0.0001$), and the association between cancer and location ($P = 0.719$). Cancer with histology ($P = 0.819$) was not statistically significant.

The endoloop was used as a prophylactic measure to prevent postpolypectomy bleeding in four cases (0.3%) for pedunculated polyps. The “endoloop” prevented bleeding from the stalk in all cases. Procedural bleeding occurred in 1.3 % (17/1354) of all polyps (11 sessile and 6 pedunculated). In large polyps the bleeding occurred in 10/160 (6.3%). Bleeding was always managed by endoscopic means with the application of hemoclips in 3 cases, adrenalin injection in 13 cases and APC in 1 case. There was no acute or late bleeding. There was no need for blood transfusion.

With the application of the Pearson χ^2 test, the association between the studied variables was shown to be statistically significant: bleeding and extra intestinal diseases ($P < 0.0001$) and the histology ($P = 0.016$). In particular, between bleeding and cardiac diseases ($P = 0.034$) and tubular adenoma ($P = 0.016$). The Pearson χ^2 test was also applied in the sub-groups for evaluation of the association between the bleeding and the studied variables and the P -values are illustrated in Table 3.

Statistical analysis performed by NPC test showed a highly significant difference between the four groups examined in relation to bleeding; the P -values of the analysis are shown in Table 4.

The results of the Binary Logistic Regression (Table 5) allowed confirmation that the associated extra intestinal diseases, histology and the size of the polyps are statistically significant and are linked to the occurrence of bleeding. For the aforesaid model the Log-Likelihood test assumes a value of -62.662, the G test is equal to 57.276 with 9 degrees of freedom and the P -value is 0.000; for this reason we can affirm that the chosen model is adequate to examine the data.

Perforation occurred in one patient (0.07%) after polypectomy of a malignant sessile polyp in the sigmoid colon, but in this lesion of 15 mm there was no suspicion of malignancy. This patient died after surgery repair of perforation from respiratory failure.

Table 4 Results of NPC test of the four groups examined in relationship with bleeding

	P-value
Group 1 vs Group 2	0.368
Group 1 vs Group 3	0.0001
Group 1 vs Group 4	0.001
Group 2 vs Group 3	0.012
Group 2 vs Group 4	0.013
Group 3 vs Group 4	0.407

Table 5 Results of the Binary Logistic regression

	Coef	SE Coef	Z	P
Associated extra intestinal diseases	0.635	0.11	5.77	0
Histology	0.509	0.226	2.26	0.024
Size	0.0567	0.019	2.97	0.003

DISCUSSION

In the past two decades the technique of endoscopic polypectomy or mucosal resection has been significantly improved and is the most common therapeutic procedure performed in the endoscopic unit. A particular challenge is the endoscopic treatment of large polyp because the procedure is difficult and reserved for experts since complications rates are very high. In this study, we described a series of 1354 endoscopic resections of colon rectal polyps performed in our endoscopic units between January, 1996 and May, 2006. The gender and age distribution of patients is similar to that described above in other studies regarding the treatment of colorectal polyps^[3-19].

From the literature, it emerges that over 80% of polyps resected during colonoscopy are small polyps less than 10 mm in diameter^[24] while in our study 67% were 10 mm in diameter. In fact, during a ten-year period we found 11.8% of excised polyps to be 20 mm or more in size out of 1354 total polypectomies. These data illustrate an average of 16 large polyps removed annually. Other authors reported averages of between 8 and 21 large polyps removed annually^[3-19]. In the present study, all patients with large colorectal polyps were treated endoscopically and in all cases the complete removal of all pedunculated and sessile polyps was possible.

Several previous publications reported a correlation between malignancy and age of patients, polyp shape, histology, location and higher rates in large polyps up to 50%^[6,25-31]. In the present study, 28 polyps were found to be adenomas containing an area of carcinoma (2.1%) and of these, 20 (12.5%) were in the group of large polyps; however, none of these polyps showed neither vascular or lymphatic invasion.

Statistical analysis, performed by biserial correlation coefficient and the χ^2 test, showed a correlation between cancer, polyp size ($P < 0.0001$), sex ($P < 0.0001$) and sessile shape ($P < 0.0001$). In accordance with previous publications, we documented a correlation with size, sessile shape and sex. Moreover, our data suggest that invasive carcinoma can appear with an equal probability, both in a

Table 6 Recent reports of series on large polyp endoscopic resection and our series

Authors	Total (n)	Pedunculated (n)	Sessile (n)	Hemorrhage (%)	Perforation (%)	Other (%)
Brooker JC <i>et al</i> ^[3]	34	-	34	17.6	0	5.9
Hsieh YH <i>et al</i> ^[4]	13	-	13	0	0	0
Brooker JC <i>et al</i> ^[5]	100	-	100	3	0	1
Walsh RM <i>et al</i> ^[6]	117	-	117	8.5	0.8	0.8
Iishi H <i>et al</i> ^[7]	56	-	56	7	0	0
Zlatanovic J <i>et al</i> ^[8]	77	-	77	6.5	1.3	0
Kanamori T <i>et al</i> ^[9]	33	-	33	9.1	0	0
Boix J <i>et al</i> ^[10]	74	-	74	13.5	0	0
Bedogni G <i>et al</i> ^[11]	66	20	42	3.1	0	1.5
Binmoeller K <i>et al</i> ^[12]	176	47	129	24	0	0
Webb WA <i>et al</i> ^[13]	102	72	30	7.8	0	0
Nivatvongs S <i>et al</i> ^[14]	280	196	84	0.7	0	1.8
Perenz RF <i>et al</i> ^[15]	147	73	74	5.4	1.3	0
Dell'Abate P <i>et al</i> ^[16]	104	49	55	3.8	0	0
Jameel JK <i>et al</i> ^[17]	30	6	24	6.1	0	0
Doniec JM <i>et al</i> ^[18]	186	45	141	15	0.5	0
Stergiou N <i>et al</i> ^[19]	68	27	41	22.1	0	0
Consolo P <i>et al</i>	160	77	83	6.2	0	0

tubular or tubulovillous or villous adenoma. Finally, there was no correlation with the site of the polyp along the colon.

Although the complications of polypectomy widely vary as presented in literature, the most frequent remain haemorrhage and perforation, which are often related to the size of the polyp, its morphology (sessile or pedunculated) and location^[14,32]. The incidence of bleeding during and after the polypectomies has been reported to range from 0.3% to 6.1%, with higher rates in large polyps up to 22.1% (Table 6)^[3-19].

In our series, the Pearson χ^2 test applied in the subgroups found a correlation between the bleeding and liver diseases ($P = 0.003$), which usually impairs the coagulation function of the patients and location of polyps in transverse colon ($P < 0.0001$), in group 2 with histology and in particular with the hyperplastic polyps ($P = 0.023$) and the malignancy ($P < 0.0001$). In addition, in group 3 bleeding was correlated with liver diseases ($P = 0.001$) and in group 4 the correlation was between the bleeding and associated colon rectal cancer ($P = 0.036$) (before or after surgical resection).

Moreover, the statistical analysis performed by NPC showed that bleeding was related to polyp size because the large polyps bled more than small polyps. These results were confirmed by Binary Logistic Regression. However, the 17 procedural bleeding that we recorded were endoscopically resolved without surgery or blood transfusion, so we can, therefore, consider these cases "non important complications". In large polyps, the rate of bleeding observed, 6.2%, is similar to that reported in the greater number of the studies (Table 6). Nevertheless, this data cannot be considered as a real complication, due to the immediate resolution during the same procedure, by endoscopic means.

The incidence of perforation during therapeutic colonoscopies has been reported to range from 0.08%-2.2%^[33]. The reported incidence of perforation during polypectomy of "normal-sized" polyps ranges from 0.3%-0.5%^[34], while the incidence of injury to the colon wall

(transmural burn, microperforation, or free perforation) in large polypectomies is 0 to 1.3% (Table 6)^[3-19].

In our study, no perforation was reported in the group of large polyps while the only late perforation (0.07%) occurred, after polypectomy, in a small (15 mm) malignant sessile polyp located in the sigmoid colon and without endoscopic sign of suspected malignancy. During the removal of this polyp, a procedural bleeding occurred, which was immediately managed by application of two hemoclips and the patients' hospitalization. Twenty four hours after the polypectomy, the patient developed lower left quadrant pain, tenderness and radiographic evidence of free air in the peritoneal cavity. The patient underwent surgery for a small (1-2 mm) perforation of the sigma. Unexpectedly, this patient died four days after surgery from respiratory failure secondary to a pleural mesothelioma, unacknowledged and diagnosed the during post mortem examination.

Summarizing, we can conclude that this study, confirming the findings of several others, demonstrates that the endoscopic polypectomy, performed by an expert hand, is safe and effective and should be considered the treatment of choice also for large colorectal polyps.

Polyp size has been identified as an important risk factor for both malignancy and bleeding. Haemorrhage was the most frequent complication even if it remains questionable whether the bleeding should be considered a complication when it occurs during the procedure and is effectively and immediately controlled by endoscopic means. Similarly, in fact, if during a surgical procedure an artery is sectioned and bleeding, this event is not considered a procedural complication because it is treated successfully. It seems likely there will be, in the upcoming future, a growing need for redefinition of the concept of endoscopic operative complications.

COMMENTS

Background

The endoscopic polypectomy is a procedure of choice for non-surgical treatment

of polyps and pre-neoplastic lesions in human colon. The most common complications of polypectomy are early or late bleeding and perforation, frequently correlated to the size of polyps and their eventual coexisting early malignancy.

Research frontiers

To investigate whether there are new possible risk factors which can be related to or influence the incidence of post-polypectomy complications type and rate.

Innovations and breakthroughs

As others, even the present study emphasizes that the endoscopic remotion of large polyps represents a challenging procedure because of its difficulty, thus should be limited to experienced endoscopists due to very high complication rate. Nevertheless, the endoscopic polypectomy in expert hands is safe and effective, and should be considered the treatment of choice both for small and for large colorectal polyps. The modality of post-procedural complications and their better definition are warranted.

Applications

Clinical application: the correlation between the incidence of bleeding and the presence of cardiovascular or liver disease stressed the importance of pre-defining and treating comorbidities in patience undergoing endoscopic resection of colonic polyps, no matter their size or supposed histology.

Peer review

This is an interesting article that reports the efficacy and complications of colonoscopic resection of colorectal polypoid lesions. This study indicated that the endoscopic resection is a simple and safe procedure for removing colon rectal neoplastic lesions.

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CLINICAL RESEARCH

Intraoperative ultrasound as an educational guide for laparoscopic biliary surgery

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educational program using IOUS is expected to minimize the incidence of BDI following LC, especially when performed by less-skilled surgeons.

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Key words: Intraoperative ultrasound; Cholecystolithiasis; Laparoscopic cholecystectomy; Bile duct injury; Education program

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Abstract

AIM: To analyze the efficacy of routine intraoperative ultrasound (IOUS) as a guide for understanding biliary tract anatomy, to avoid bile duct injury (BDI) after laparoscopic cholecystectomy (LC), as well as any burden during the learning period.

METHODS: A retrospective analysis was performed using 644 consecutive patients who underwent LC from 1991 to 2006. An educational program with the use of IOUS as an operative guide has been used in 276 cases since 1998.

RESULTS: IOUS was highly feasible even in patients with high-grade cholecystitis. No BDI was observed after the introduction of the educational program, despite 72% of operations being performed by inexperienced surgeons. Incidences of other morbidity, mortality, and late complications were comparable before and after the introduction of routine IOUS. However, the operation time was significantly extended after the educational program began ($P < 0.001$), and the grade of laparoscopic cholecystitis ($P = 0.002$), use of IOUS ($P = 0.01$), and the experience of the surgeons ($P = 0.05$) were significant factors for extending the length of operation.

CONCLUSION: IOUS during LC was found to be a highly feasible modality, which provided accurate, real-time information about the biliary structures. The

INTRODUCTION

Laparoscopic cholecystectomy (LC) is widely accepted as a standard treatment for symptomatic cholecystolithiasis. However, the incidence of complications in the form of bile duct injury (BDI) is high, and it has been reported to be as high as twice the normal rate^[1,2]. Once such BDI occurs, it increases postoperative morbidity^[3] and mortality^[4,5], and decreases long-term quality of life^[6], which consequently results in a high number of lawsuits^[7]; therefore, it should be prevented if at all possible^[1,8]. It is necessary to have a sufficient understanding of the biliary anatomy to prevent BDI^[1], yet the damage is often caused by misidentification of the bile duct and other normal structures^[9-11]. Therefore, the inappropriateness of a procedure is often not discovered until the bile duct has already been injured.

To prevent BDI, intraoperative guidance by intraoperative cholangiography and intraoperative ultrasonography (IOUS) has been suggested. Although cholangiography is effective in diagnosing biliary tract injury, it remains controversial as to whether the routine implementation of cholangiography will prevent BDI^[2,12-15]. Moreover, intraoperative cholangiography requires a significant amount of hospital treatment to prevent BDI^[16]. On the other hand, IOUS requires a shorter examination time^[17-19], is safe without incurring radiation exposure,

and is minimally invasive^[18,20]. It has been reported that IOUS allows for visualization equal to or better than that of cholangiography^[17,20-23] in the case of biliary anatomy and diagnosis of bile duct stones. However, there have been few studies that have investigated whether the routine implementation of IOUS decreases BDI during LC^[24]. Moreover, a sufficient learning period is considered necessary for IOUS^[25,26]. However, there have been very few studies on visualization of biliary anatomy, diagnostic performance, or changes in decision-making during the learning period in less-skilled surgeons, or on the extension of operation time caused by the introduction of IOUS.

Therefore, in this study, IOUS was routinely introduced to an educational program for laparoscopic biliary surgery, to analyze its efficacy in helping surgeons avoid biliary tract injury and other complications, and to analyze the extension of operation time during the learning curve.

MATERIALS AND METHODS

Patients

Among 664 patients who underwent laparoscopic biliary surgery at the Hirosaki University Hospital from March 1991 to December 2006, 644 were targeted after excluding 20 in whom surgery other than biliary tract surgery was performed. Senior surgeons with experience in laparoscopic surgery in at least 100 cases were in charge from 1991 to 1997 as a rule, but the educational program for laparoscopic biliary surgery began in 1998 to teach young surgeons with experience of less than 30 LCs. Furthermore, IOUS using a linear probe was introduced in October 1992 for the purpose of making an intraoperative diagnosis and as a guide for surgical procedures at the start of a cholecystectomy. IOUS was used on a sporadic basis at first, but it became routine at the start of the educational program in 1998.

Endoscopic retrograde cholangiopancreatography was performed preoperatively on selective cases with icterus or a high level of serum transaminases. Moreover, intraoperative cholangiography was selectively adopted in the earlier period when bile duct stones were suspected by preoperative examination, and was selectively performed in the later period when bile duct stones or variations in biliary distribution were suspected *via* IOUS.

We included 368 cases before the introduction of the educational program and 276 cases after its introduction (Table 1). There was no gender difference. Although the age was significantly higher in the latter term, all cases underwent elective surgery after sufficient assessment of their general health status. There was no significant difference in the indicated disorders for surgery, and cholecystolithiasis accounted for at least 80% of the cases. The rate of coexisting biliary infection and the diameter of the bile duct measured in the preoperative examination were comparable between the two periods.

These cases were retrospectively assessed for postoperative morbidity and mortality, operation time, and length of hospital stay, as well as late outcomes before and after the introduction of the educational program that adopted IOUS as a routine operative guide.

Table 1 Patient demographic data

	Before routine IOUS (1991-1997)	Routine IOUS (1998-2006)	P
Patient number (n)	368	276	
Male/Female	140/228	107/169	0.85
Age (yr)	53.3 ± 13.9	57.1 ± 13.6	0.004
Operative indications, n (%)			0.73
Cholecystolithiasis	296 (80.4)	228 (82.6)	
Cholecystocholedocholithiasis	39 (10.6)	23 (10.5)	
Choledocholithiasis	7 (1.9)	4 (1.4)	
Gallbladder polyp	22 (6.0)	11 (4.0)	
Others ¹	4 (1.1)	4 (1.4)	
Symptomatic gallstones, n (%)	284 (77.2)	220 (79.7)	0.44
Acute cholecystitis/choolangitis, n (%)	42 (11.4)	32 (11.6)	0.94
Bile duct diameter ² (mm)	8.9 ± 4.2	8.4 ± 3.6	0.12

¹Other benign processes including chronic cholecystitis and adenomyomatosis, which need whole biopsy of the gallbladder for suspected malignancy.

²Maximal dimension of the extrahepatic bile duct was measured by drip infusion cholangiography (51%), endoscopic retrograde cholangiography (35%), percutaneous transhepatic cholangiography (4%), magnetic resonance cholangiography (2%), and/or extracorporeal ultrasonography (8%).

For statistical analysis, Student's *t* test, χ^2 test, and analysis of covariance were used appropriately, and the SPSS 11.0 for Windows was used for the analysis. Statistically, it was determined that $P < 0.1$ indicated a tendency and $P < 0.05$ indicated a significant difference.

Procedure of IOUS

A deflectable sonographic probe with a linear head of 7-7.5 MHz LAP-703LA (Toshiba-Mochida, Tokyo, Japan), PEF-704LA (Toshiba, Tokyo, Japan) and MH-300 (Olympus, Tokyo, Japan) were used. After pneumoperitoneum, a probe was inserted from a port on the left upper abdomen, and the entire extrahepatic biliary tract from the hepatic hilum to the biliary terminal was scanned, before initiating an exfoliating procedure around the gallbladder. The presence or absence of visualization of the right and left hepatic duct, the common hepatic duct, the common bile duct, the intrapancreatic bile duct, the gallbladder, the cystic duct, and the right hepatic artery were recorded as video recordings and photographs. Next, an intraoperative diagnosis for surgery was made by creating both transverse and longitudinal ultrasonographic images of the gallbladder and the bile duct. When the anatomy at the confluence of the extrahepatic bile duct and the cystic duct could not be sufficiently confirmed due to either acute cholecystitis or adhesion, IOUS was performed during and after the exfoliating procedure and before clipping to avoid duct injury.

In the earlier period, senior surgeons performed IOUS. In the later period, when a senior surgeon performed the operation, a demonstration of the IOUS procedure was given to a junior surgeon. On the other hand, when a junior surgeon performed the operation, they were required to perform IOUS on their own, to understand the biliary anatomy and to make a correct diagnosis. The senior surgeon gave appropriate suggestions or aids, if necessary, according to the junior surgeon's skill.

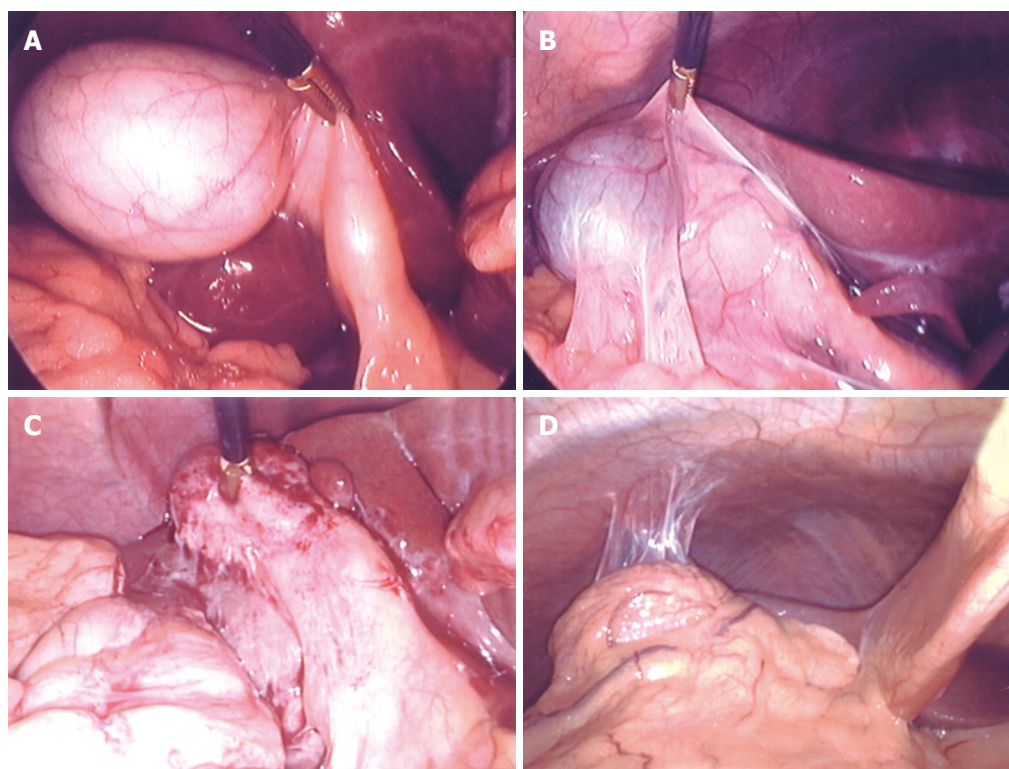


Figure 1 Laparoscopic cholecystitis grading. **A:** Normal (G0); **B:** No inflammation with light adhesion (G1); **C:** Marked wall thickening with light adhesion (G2); **D:** Marked inflammation with dense adhesion (G3). Classification of severity of cholecystitis was based on laparoscopic findings to assess the effect of the presence of cholecystitis on intraoperative diagnostic performance and operative performance.

Laparoscopic cholecystitis grading

Inflammation around the gallbladder affects the visual field in laparoscopic surgery, as well as the level of difficulty of a surgical procedure. Moreover, it may affect diagnostic ability of IOUS. Therefore, laparoscopic cholecystitis grading (LCG) was suggested, based on macroscopic findings at the start of the laparoscopic surgery. The severity of cholecystitis was classified into four grades: G0, normal; G1, no acute inflammation with old fibrous adhesion; G2, marked inflammatory wall thickening with light adhesion; and G3, unidentifiable gallbladder due to dense inflammatory adhesion of the surrounding tissues (Figure 1).

RESULTS

In the earlier period, senior surgeons performed surgery in 93% of cases, while junior surgeons performed 72%. The average number of operations per surgeon was 5.1 in the later period. IOUS was performed in 19% of cases in the earlier period, but after introduction of the educational program, it was performed in 84% of cases. There was no significant difference in the frequency of anatomical variations of the biliary tree between the two groups (2.7 *vs* 4.3%, $P = 0.26$). According to the cholecystitis grade as determined under laparoscopic view, the degree of cholecystitis was different between the two periods ($P = 0.03$); the percentage with G2 disease was higher in the earlier period, while G3 was higher in the later period. The number of conversions to open surgery due to cholecystitis or adhesion significantly increased in the later period ($P = 0.007$). In the earlier period, there were two cases of BDI treated with hepaticojejunostomy, while in the later period, no patients underwent laparotomy for BDI.

After the educational program began, the operation time increased by an average of 23 min in patients that were treated laparoscopically. There was no difference in surgery for bile duct stones, but in the later period, there was a significant extension of operation time in patients who underwent cholecystectomy ($P < 0.0001$). On the other hand, there was no significant difference in the amount of blood loss between the two groups (Table 2).

An analysis of covariance conducted on the prescribed factors of operative time showed that the degree of LCG ($P = 0.002$), presence or absence of implementation of IOUS ($P = 0.01$), and experience of the surgeon ($P = 0.05$) were significant factors for an extension of operation time after introduction of the educational program. No confounding effect was observed for these three factors (Table 3). The relationship between these three factors and the operation time is shown diagrammatically in Figure 2. The operation time was extended as LCG became more severe, and junior surgeons required more time for IOUS for all LCG compared with senior surgeons. The extension of operation time caused by implementation of IOUS for senior surgeons in the earlier period was 12.7 min (78.2 ± 37.8 min with IOUS *vs* 90.9 ± 32.4 min without IOUS, $P = 0.02$), while the average extension due to the use of IOUS for junior surgeons was 20.9 min (94.3 ± 43.6 min *vs* 115.2 ± 40.2 min, $P = 0.003$).

Regarding the frequency of early intraoperative and postoperative complications, the number that required re-operation, and mortality, there was no significant difference between the two periods, despite the fact junior surgeons performed many operations in the later period. Moreover, there were no cases of BDI after introduction of IOUS, and a decreasing tendency was observed ($P = 0.08$). The length of hospital stay was shortened in the later period,

Table 2 Various operations before and after routine IOUS

	Before routine IOUS (<i>n</i> = 368)	Routine IOUS (<i>n</i> = 276)	<i>P</i>
Surgeon, <i>n</i> (%)			
Senior (≥ 100 cases)	341 (92.7)	77 (27.9)	< 0.0001
Junior (< 30 cases)	27 (7.3)	199 (72.1)	
Intraoperative image studies, <i>n</i> (%)			
Ultrasound	68 (18.5)	231 (83.7)	< 0.0001
Cholangiography	45 (12.7)	25 (9.1)	
Anatomical variations of the bile ducts, <i>n</i> (%)	10 (2.7)	12 (4.2)	
Laparoscopic cholecystitis grading, <i>n</i> (%)			
G0	203 (55.2)	166 (60.1)	0.03
G1	59 (16.0)	40 (14.5)	
G2	72 (19.6)	33 (12.0)	
G3	34 (9.2)	37 (13.4)	
Conversion to open surgery, <i>n</i> (%)	6 (1.6)	15 (5.4)	0.007
BDI	2	0	0.27
Severe cholecystitis	2	4	
Cholecystoduodenal fistula	0	1	
Access failure due to dense adhesion	1	5	
Cancer suspected	1	1	0.007
Systemic co-morbidity	0	3	
Total laparoscopic procedures, <i>n</i> (%)	362 (98.4)	261 (94.6)	0.34
Cholecystectomy	317 (87.6)	235 (90.0)	
Cholecystectomy and choledocholithotripsy	45 (12.4)	26 (10.0)	
Operative time ¹ (min)	93.7 \pm 53.1	116.7 \pm 49.7	< 0.0001
Cholecystectomy	80.6 \pm 36.2	109.4 \pm 42.1	
Cholecystectomy and choledocholithotripsy	185.4 \pm 62.0	187.6 \pm 61.2	
Blood loss ¹ (g)	16.9 \pm 75.5	14.1 \pm 53.2	0.6
Cholecystectomy	10.1 \pm 42.8	15.1 \pm 55.4	
Cholecystectomy and choledocholithotripsy	65.1 \pm 175.4	4.2 \pm 20.4	

¹In cases treated with total laparoscopic procedures.

Table 3 Factors determining operation time of LC

Factors	<i>F</i> value	<i>P</i> value
Use of IOUS	6.524	0.011
Operative indication	1.071	0.370
Presence of cholecystitis symptom	2.381	0.123
Surgeon (senior/junior)	3.778	0.053
Intraoperative cholangiography	1.685	0.195
LCG: G0/G1/G2/G3	5.148	0.002
IOUS \times Surgeon	0.208	0.648
IOUS \times LCG	0.359	0.782
Surgeon \times LCG	0.424	0.736

but it was assumed that a major reason for this included changes in the clinical pathway applied. There was no late bile duct stenosis in the later period (Table 4).

The BDI that occurred in the earlier period included three cases of complete disjunction of the bile duct. All cases were identified for the first time after the bile duct was damaged. In Case 1, the bile duct was severed due to

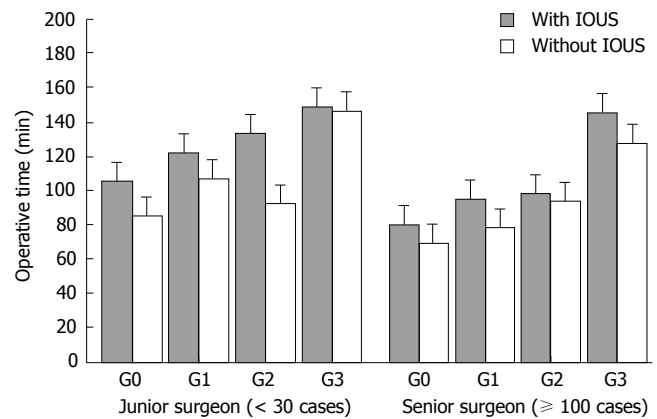


Figure 2 Operative time for LC. In an analysis of covariance, LCG, as well as presence and absence of implementation of IOUS, were significant factors that affected operation time. Operation time when IOUS was implemented significantly increased for junior surgeons (all, $P < 0.01$), regardless of LCG, thus requiring approximately double the time in comparison to that of senior surgeons.

Table 4 Early and late results

Outcome	Before routine use (<i>n</i> = 368)	Routine use (<i>n</i> = 276)	<i>P</i>
Early complication, <i>n</i> (%)			
BDI	4 (1.1)	0	0.08
Arterial bleeding requiring clips	9 (2.4)	5 (1.8)	0.59
Postoperative bleeding	2 (0.5)	1 (0.4)	0.74
Surgical site infection	1 (0.3)	1 (0.4)	0.84
Re-operation	1 ¹ (0.3)	1 ² (0.4)	0.84
Hospital death	1 ³ (0.3)	0	0.39
Postoperative treatment for duct stone	7 ⁴	2 ⁵	0.19
Hospital stay (d)	5.4 \pm 5.7	4.4 \pm 2.6	0.006
Late complication (> 1 yr), <i>n</i> (%)			
Bile duct recurrence	1 (0.3%)	0	0.39
Bile duct stricture	0	0	-

¹Open hemostasis of postoperative bleeding from the liver bed; ²Laparoscopic hemostasis of a small arterial bleeding at the port site; ³Brain stem infarct on POD 4; ⁴All treated with percutaneous cholangiofiberscopic lithotripsy through preoperatively-established sinus tracts; ⁵Both treated with endoscopic sphincterotomy.

misidentification of the cystic duct, but it was caused by a variant distribution of the cystic duct and the extrahepatic bile duct. In Case 2, because tenting occurred in the common bile duct due to pulling of the gallbladder, the bile duct was misidentified as the cystic duct. Case 3 was caused by variation in the distribution of the biliary tract where the cystic duct joined the accessory hepatic duct. In the remaining case, a small fissure occurred in the hepatic duct during an exfoliating procedure of the severely inflamed gallbladder, and was treated with simple suturing. Furthermore, hepatocholejojunostomy was performed during laparotomy in two cases (Table 5).

Regarding the 299 cases in which IOUS was performed, anatomical visualization of IOUS was 100% for the gallbladder, 97.3% for the extrahepatic bile duct, 96.3% for the pancreatic bile duct, 98.5% for the gallbladder duct, and 98.4% for the right hepatic artery.

Table 5 BDI during LC

	Age (yr)/gender	Indication	Anatomical variation	LCG	Type of injury	Repair
1	58/female	Cholecystolithiasis	+ ¹	2	Cutting of the common hepatic duct	Hepaticojejunostomy (Roux-en-Y)
2	43/female	Cholecystolithiasis	-	0	Cutting of the common hepatic duct	Hepaticojejunostomy (Roux-en-Y)
3	86/female	Chronic cholecystitis (cancer suspected)	+ ²	0	Cutting of the posterior branch of the right hepatic duct	Duct-to-duct anastomosis using an internal stent
4	63/female	cholecystocholedocholithiasis	-	3	A small tear of the common hepatic duct at the dense adhesion to the neck of the gallbladder	Simple suture

¹The cystic duct ran behind the common hepatic duct and connected with it at the left lateral wall near the pancreas; ²The cystic duct joined the posterior branch of the right hepatic duct.

Table 6 Diagnostic accuracy of IOUS (%)

	Sensitivity		Specificity		PPV		NPV		Overall accuracy	
	Pre-op	IOUS	Pre-op	IOUS	Pre-op	IOUS	Pre-op	IOUS	Pre-op	IOUS
Anatomical variation	25	100	100	100	100	100	96	100	96	100
Cholecystolithiasis	99	100	100	100	100	100	87	100	99	100
Bile duct stone	76	76	100	99	100	93	95	95	96	95
Polyps	77	82	98	98	77	75	98	99	97	97
Others	50	81	93	95	70	82	86	94	83	92

PPV: Positive predictive value; NPV: Negative predictive value; Pre-op: Preoperative image studies; Anatomical variation: Anatomical variation of the extrahepatic bile duct. Preoperative image studies included extracorporeal ultrasonography (100%), computed tomography (73%), endoscopic retrograde cholangiopancreatography (51%), drip infusion cholangiography (42%), magnetic resonance cholangiopancreatography (20%), and/or percutaneous transhepatic cholangiography (5%).

The diagnostic performance of IOUS regarding the indicated disorders was equal to preoperative diagnostic imaging, but 10 cases determined to have no bile duct stones *via* preoperative diagnostic imaging were diagnosed to have complications of choledocholithiasis *via* IOUS, which turned out to be useful for making changes in the operation policy (Table 6). Biliary tract anatomy was visualized in almost all patients with G0-G2 laparoscopic cholecystitis, whereas visualization remained at 88% for G3 disease (Table 7).

Table 7 Ultrasonographic visualization of the biliary and arterial structures according to the laparoscopic cholecystitis grading (%)

	G0	G1	G2	G3	P value
Gallbladder	100	100	100	100	-
Cystic duct	100	95.6	100	91.4	0.001
Bifurcation	97.1	95.6	93.3	88.6	0.15
Common hepatic duct	99.4	95.6	97.8	88.6	0.003
Intrapancreatic bile duct	98.3	97.5	93.3	88.6	0.03
Right hepatic artery	100	97.5	100	89.3	0.0004

DISCUSSION

In the educational program for laparoscopic biliary surgery with IOUS as a guide, no cases of BDI were observed, despite the fact that less-skilled surgeons performed the operation in 72% of patients.

BDI is mostly caused by anatomical misidentification of the bile duct^[9-11]. In addition, it is frequently caused by less-skilled surgeons^[27,28]. The reasons for this misidentification are that laparoscopic surgery is performed in a two-dimensional world without any touch sensation, and that young surgeons have fewer opportunities to understand the anatomy of the biliary tract in abdominal surgery. Therefore, auxiliary means to improve understanding of the biliary tract anatomy is necessary to avoid BDI.

Cholangiography is an effective method for understanding biliary tract pathology^[2,5,14]. However, its preventive effect against BDI is controversial^[12,13,15], because a randomized control trial has not yet been conducted. Moreover, cholangiography has an unavoidable risk that examination can cause ductal injury during cannulation. Furthermore, there is the problem that BDI

cannot be prevented in patients in whom the cystic duct merges into the accessory bile duct, as in our Case 3.

IOUS, on the other hand, is a less-invasive procedure than cholangiography. It can be performed in most cases more quickly and repeatedly, without any damage to the biliary tree^[17-20]. Moreover, it does not require new X-ray apparatus, radiologists, or laboratory technicians. Many studies, including two randomized control trials, have reported that visualization of the biliary tract anatomy and diagnostic performance of IOUS for bile duct stones are as high, or better, than those for cholangiography^[17,21-23]. It has also been reported that IOUS decreases the necessity to perform cholangiography^[29,30].

However, it is necessary to learn properly and fully the technique of implementing IOUS^[25]. Falcone *et al*^[26] have reported it is necessary to learn the procedure from at least 10 cases. While there are many reports on the efficacy of IOUS, there has been no analysis on the clinical outcome and the time burden in these learning curve periods needed for IOUS.

Therefore, we introduced routine IOUS to laparoscopic

surgery for the purpose of educating less-skilled surgeons regarding the anatomy of the biliary tract and avoidance of BDI. IOUS was able to be implemented in all cases, and the visualization ability for the extrahepatic bile duct was almost 100%. The diagnostic sensitivity for abnormal distribution of the biliary tract also significantly improved to 100% through the concomitant use of IOUS, from 25% by preoperative examinations only. Consequently, an abnormal distribution or tenting of the biliary tract was identified in all of our patients before the exfoliating procedure and other invasive procedures such as clipping of the cystic duct, so BDI was avoided. Moreover, the incidence rate of postoperative morbidity and mortality in the latter stages did not increase, despite operations being performed by less-skilled surgeons.

While IOUS has high feasibility, there is a concern that inflammatory processes around the gallbladder may reduce visualization of the biliary anatomy. Therefore, an LCG system was suggested to study the visualization rate of the biliary structures according to the degree of cholecystitis. In G0-G2, the confluence of the cystic and bile ducts was confirmed within the hepatoduodenal ligament in almost all cases. Biliary tract injury due to misidentification occurs frequently in cases without or with mild-to-moderate cholecystitis, therefore, IOUS is expected to have an effect in preventing biliary tract injury. On the other hand, for severe cholecystitis (G3), the gallbladder was identified in all cases, but the visualization rate of the extrahepatic bile duct and cystic duct decreased to 88%. In patients in whom the bile duct was visualized *via* IOUS, laparoscopic surgery was performed safely, but in cases in which the bile duct could not be identified, insufficient identification of the bile duct was determined to be the reason for conversion to laparotomy. Therefore, the number of cases converting to a laparotomy increased in the later period, which had a higher percentage of G3 disease.

After the introduction of the educational program for laparoscopic biliary surgery, the operative time became significantly extended. Higher LCG, implementation of IOUS, and performance by an inexperienced surgeon were independent factors that significantly extended the operation time. The operation time with a junior surgeon was extended by an average 20.9 min, after implementation of IOUS, which was approximately double the time in a previous study of 10 min^[17]. In addition to the technical demonstration of IOUS by senior surgeons, junior surgeons were required to perform IOUS again and record all the biliary structures on a check list. Moreover, the extended time required for IOUS implementation was calculated by measuring the total operation time, which included the time required for preparation and recording of IOUS, unlike in previous studies in which only the time of implementing IOUS was recorded^[17,19,22]. Consequently, a longer period of time was recorded in this study, especially when junior surgeons operated.

In any educational programs, the burden on the patient must be within acceptable limits. The extended time required for IOUS is believed acceptable compared with the time required for cholangiography^[17-20], along with the advantage of increasing the possibility of avoiding

biliary tract injury. Of course, it is necessary to analyze the economic efficiency of the introduction of IOUS. The system of paying medical expenses in Japan has largely changed during the past 10 years, therefore, it is difficult to directly compare expenses. However, after the introduction of this educational program, the frequency of complications did not increase, and the frequency of BDI tended to decrease. Therefore, an extension of operation time was the only economic disadvantage, and the increase in expenses was moderate. Furthermore, the amount invested in probes for IOUS is small, and cost effectiveness is higher than that of cholangiography.

This study had prospective data collection but it did not constitute a randomized control trial. Moreover, because the decrease in frequency of BDI remained at $P = 0.08$, the benefit of the education program for using IOUS cannot be concluded. Furthermore, to confirm the educational outcomes, it is necessary to conduct a follow-up review on the performance of subsequent laparoscopic biliary tract surgery performed by a physician who has completed the educational program. It will therefore be necessary to assess the benefits of this educational program for laparoscopic biliary surgery using IOUS after more extensive randomized control trials.

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COMMENTS

Background

Bile duct injury (BDI) following laparoscopic cholecystectomy (LC) occurs at a rate twice that observed after open surgery, and misidentification of the biliary tract structures is the major cause of BDI. Intraoperative ultrasound (IOUS) is less invasive and reportedly has an equal or higher visualization ability of the biliary tract anatomy in comparison to cholangiography.

Research frontiers

In this study, the authors developed a new educational program for laparoscopic biliary surgery by introducing IOUS routinely as a guide for anatomical understanding, and analyzed its efficacy in enabling surgeons to avoid BDI and other complications, as well as any burden during the learning period.

Innovations and breakthroughs

Under the IOUS-guided educational program, no case of BDI was observed, despite the fact that less-skilled surgeons performed the operation in 72% of patients. Excess operation time was minimal. IOUS was feasible even in the presence of severe cholecystitis.

Applications

The educational program using IOUS is expected to minimize the incidence of BDI following LC, especially when performed by less-skilled surgeons, by giving them accurate, real-time information about the biliary tract structures.

Peer review

This study is very interesting, as it appeared to explore a good alternative to intraoperative cholangiography. In general, this clinical research was well performed and the results were convincing.

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Changes in count and function of splenic lymphocytes from patients with portal hypertension

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Abstract

AIM: To investigate changes in numbers and proliferative function of splenic lymphocytes in patients with hypersplenism due to portal hypertension (PH), to provide evidence for further study of immune status of the spleen during PH.

METHODS: Twelve spleens from patients with hypersplenism due to PH served as the PH group, and four spleens from cases of traumatic spleen rupture were regarded as the control group. After weighing the spleen, lymphocytes were separated and counted using a cell counting plate to calculate the lymphocyte count per gram of spleen tissue (relative quantity) and total lymphocyte count in whole spleen (absolute quantity). The immunohistochemical SP method was used to observe the density and distribution of lymphocytes in the spleen. The MTT method was used to observe changes in lymphocyte proliferative function.

RESULTS: As compared to the control group, the splenic lymphocytes in the PH group showed that: (1) There was no difference in distribution but a significant decrease

in density; (2) the number of lymphocytes per gram of spleen (relative quantity) decreased significantly [$(0.822 \pm 0.157) \times 10^8$ vs $(1.174 \pm 0.254) \times 10^8$, $P < 0.01$]; (3) with the significant increase in the weight of the PH spleen (832.6 ± 278.2 g vs 211.7 ± 85.6 g, $P < 0.01$), the total quantity of lymphocytes (absolute quantity) increased significantly [$(0.685 \pm 0.072) \times 10^{11}$ vs $(0.366 \pm 0.057) \times 10^{11}$, $P < 0.01$]; and (4) the proliferative function of lymphocytes was enhanced: T lymphocytes, $(0.022 \pm 0.005$ vs 0.015 ± 0.003 , $P < 0.05$), and B lymphocytes $(0.034 \pm 0.006$ vs 0.023 ± 0.001 , $P < 0.01$).

CONCLUSION: Although lymphocyte density in the spleen decreased in patients with PH, the total quantity of lymphocytes increased because spleen weight increased greatly, along with the proliferating function. With respect to changes in lymphocytes, PH spleens may still have immune function, although it may be disordered. However, complete evaluation of the immune function of the spleen in PH requires more research.

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Key words: Portal hypertension; Spleen; Lymphocyte; Immune function

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INTRODUCTION

Lymphocytes reside in different organs in the human body. They circulate through the primary lymphoid organs (thymus and bone marrow), secondary lymphoid organs (spleen, lymph nodes, tonsils and Peyer's patches), as well as non-lymphoid organs such as blood, lung and liver. Especially in lymphoid organs, lymphocyte subsets migrate and home to different compartments. About 15%-20% of the blood volume circulates through the spleen at any one time and about 15% of the lymphocytes reside in this organ^[1,2].

Therefore, lymphocytes are the immunocytes that have the highest count in the spleen. Their functional status directly influences the immune function of the spleen^[3-5].

Currently, there is still some dispute on the immune status of the spleen in patients with portal hypertension (PH)^[6,7]. We have isolated splenic macrophages and demonstrated that their phagocytosis is enhanced in PH spleens^[8-10], but there is little compelling experimental evidence on the distribution, count and function of lymphocytes in the PH spleen. In this study, we isolated and cultured splenic lymphocytes from PH spleen, and observed changes in their density, distribution, count, and proliferative function using the immunohistochemical SP method and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT).

MATERIALS AND METHODS

Patients

Two groups of patients were studied. Twelve patients (median age 46.8 years, range 27-62 years; eight male and four female), with hypersplenism due to PH, in our hospital from September 2005 to March 2006, were included as the PH group. All patients underwent pericardial devascularization with splenectomy. Supporting evidence for hypersplenism due to PH and cirrhosis included clinical features, abnormal laboratory tests, and postoperative pathological examination. Four patients (median age 33.5 years, range 18-38 years; three male and one female) with traumatic rupture of the spleen were enrolled into the control group. Hepatitis, cirrhosis, history of hypersplenism, and abnormalities in postoperative laboratory findings and pathological examinations were absent in the controls. All patients provided written informed consent, and the protocol was approved by the ethics committee of our hospital.

Lymphocyte count in the spleen

Spleens were weighed after removal from patients. The splenic tissue samples were cut from the upper pole, lower pole, and hilum, and were transferred to the cell culture room, and kept in sealed aseptic bottles filled with 4°C precooled PBS. Further preparations were made: Weighing 5 g tissue with an electronic balance, using a 200-mesh screen to grind the tissue sample into cell suspension, and purifying the lymphocytes with lymphocyte separating medium by gradient centrifugation. After preparation, lymphocytes were counted using a cell counting plate. The lymphocyte count per gram of spleen tissue (relative quantity) was then calculated and multiplied by the weight of the spleen to derive the total lymphocyte count in the spleen (absolute quantity).

Density and distribution of lymphocytes

The splenic tissue samples were cut from the upper pole, lower pole, and hilum, fixed in phosphate buffer (pH 7.2) containing 4% paraformaldehyde, embedded in paraffin wax, and sectioned at 5 µm. CD3 and CD20 SP method staining was adopted to show T and B lymphocytes, respectively. Periarterial lymphatic sheath (PALS), splenic corpuscle (F), red pulp (RP), and marginal zone (MZ) of spleen tissue were

observed. Five fields of vision were also randomly observed in each part. Positive cells were counted respectively. For the negative control, the primary antibody was replaced by PBS.

Proliferative function of lymphocytes

Lymphocytes with RPMI1640 culture solution containing 10% fetal calf serum were placed in 96-well flat-bottomed microplates in triplicate at 2×10^5 cells/well, then concanavalin A (Con A) or lipopolysaccharide (LPS; both from Sigma, St. Louis, MO, USA) was added to the wells at a final concentration of 10 µg/L and 20 µg/L, respectively. The cells were then incubated in a total volume of 200 µL/well. Serum-free RPMI-1640 medium was used as a control. Cell proliferation was measured by MTT assay 44 h after culture. MTT (Sigma) solution of 20 µL (5 g/L) was added to each well. After 4 h incubation, the cells were lysed and the purple formazan crystals were solubilized. We then measured A_{570} of each well on an enzyme labeling instrument, and the proliferation level was calculated. Proliferation level = experimental group A (ConA or LPS) - negative control group A .

Statistical analysis

P values were calculated using the independent sample t test and considered significant at $P < 0.05$. All the results were represented by mean \pm SD.

RESULTS

Change in density and distribution of lymphocytes

The distribution of T and B lymphocytes was almost the same in PH spleen as in normal spleen (Figures 1 and 2). Cell counts in single fields of view were significantly less in PH spleen than in normal spleen (Table 1, Figures 3 and 4).

Change in lymphocyte count

Lymphocyte count was significantly less in PH spleen (relative quantity) than in normal spleen. However, with the increase in spleen weight, lymphocyte count of whole spleen (absolute quantity) was significantly greater in PH spleen than in normal spleen (Table 2).

Change in lymphocyte proliferative function

The proliferative function of T and B lymphocytes was significantly higher in PH spleen than in normal spleen (Table 3).

DISCUSSION

At present, the immune function of the PH spleen is still the subject of some dispute^[7,11,12]. It is unclear if the preservation of splenic tissue with splenomegaly is beneficial to patients with PH and cirrhosis^[13-15]. The lymphocytes are important immunocytes in the spleen. The spleen can participate in specific immunity through T-cell-mediated cellular immunity and B-cell-mediated humoral immunity^[16-18]. Therefore, the evaluation of changes in lymphocyte count and functions in PH spleen is extremely important to an in-depth study of the immune status of the spleen in PH.

Table 1 Changes in density of lymphocyte in PH spleen

Group	T lymphocytes				B lymphocytes			
	F	MZ	PALS	RP	F	MZ	PALS	RP
PH	89.5 ± 14.7	120.0 ± 14.1	122.9 ± 12.1	12.2 ± 2.9	356.5 ± 31.2	138.0 ± 19.5	113.8 ± 21.6	7.4 ± 1.7
Control	126.5 ± 19.3	140.5 ± 11.6	137.0 ± 6.2	20.45 ± 4.5	418.3 ± 22.4	196.0 ± 22.0	153.8 ± 25.8	12.8 ± 4.6
P value	< 0.01	< 0.05	< 0.05	< 0.01	< 0.01	< 0.01	< 0.05	< 0.01

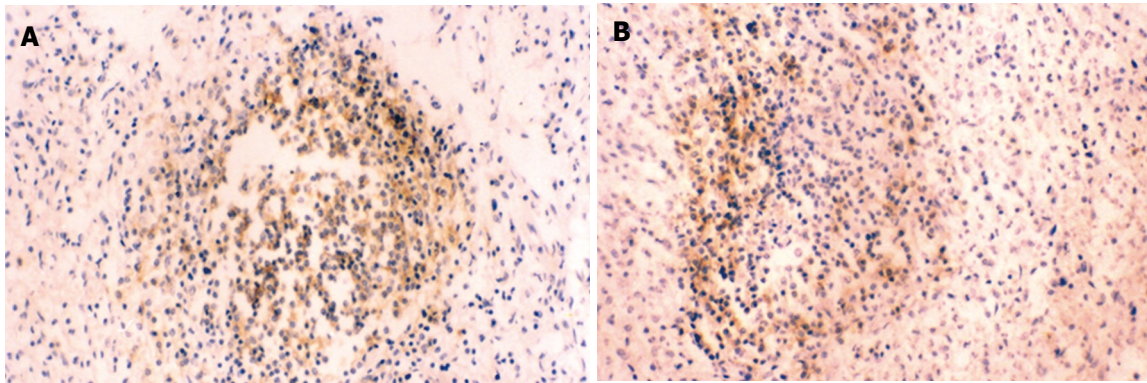


Figure 1 CD20 immunostaining for distribution of B lymphocytes. There was no significant difference in distribution of B lymphocytes between PH and control groups; they were all mainly located in the splenic corpuscle. **A:** Control group; **B:** PH group ($\times 100$).

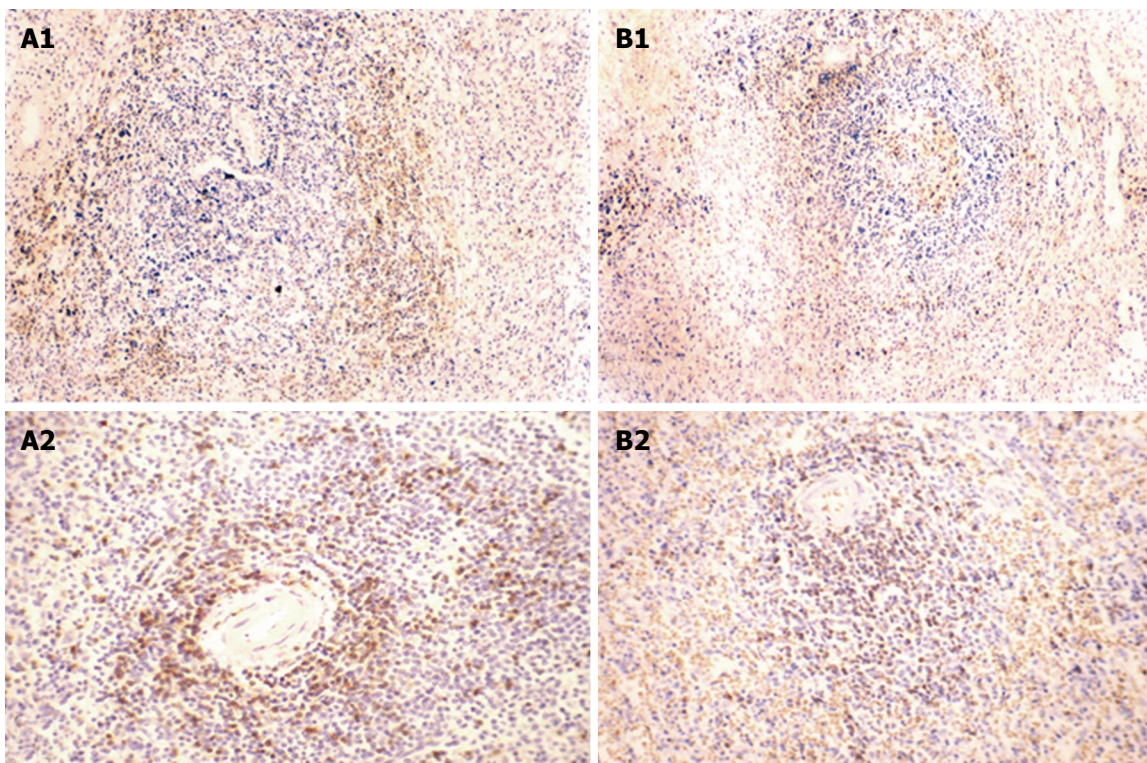


Figure 2 CD3 immunostaining for distribution of T lymphocytes. There was no significant difference in distribution of T lymphocytes between PH and control groups; they were all mainly located in the marginal zone and PALS. **A1:** Control group, marginal zone; **A2:** Control group, PALS; **B1:** PH group, marginal zone; **B2:** PH group, PALS ($\times 100$).

Lymphocytes include T and B lymphocytes. CD3 and CD20 are important differentiation antigens on T- and B-cell membranes. CD3 and CD20 immunohistochemical staining is ideal for analyzing the distribution and count of T and B lymphocytes in tissue^[19]. Wang *et al* have used the

method to observe lymphocytes in pathological sections of PH spleen. They believe that in PH splenomegaly, lymphocyte density in the spleen decreases, which results in a decrease in lymphocyte count^[20]. We also found in our experiment that the distribution area of lymphocytes had

Table 2 Changes in weight of spleen and lymphocyte count

Group	Relative quantity ($\times 10^8$)	Weight of spleen (g)	Absolute quantity ($\times 10^{11}$)
PH	0.822 ± 0.157	832.6 ± 278.2	0.685 ± 0.072
Control	1.714 ± 0.254	211.7 ± 85.6	0.366 ± 0.057
P value	< 0.01	< 0.01	< 0.01

Table 3 Changes in proliferative function of lymphocyte in PH spleen

Group	T lymphocyte (ConA $10 \mu\text{g/L}$)	B lymphocyte (LPS $20 \mu\text{g/L}$)
PH	0.022 ± 0.005	0.034 ± 0.006
Control	0.015 ± 0.003	0.023 ± 0.004
P value	< 0.05	< 0.01

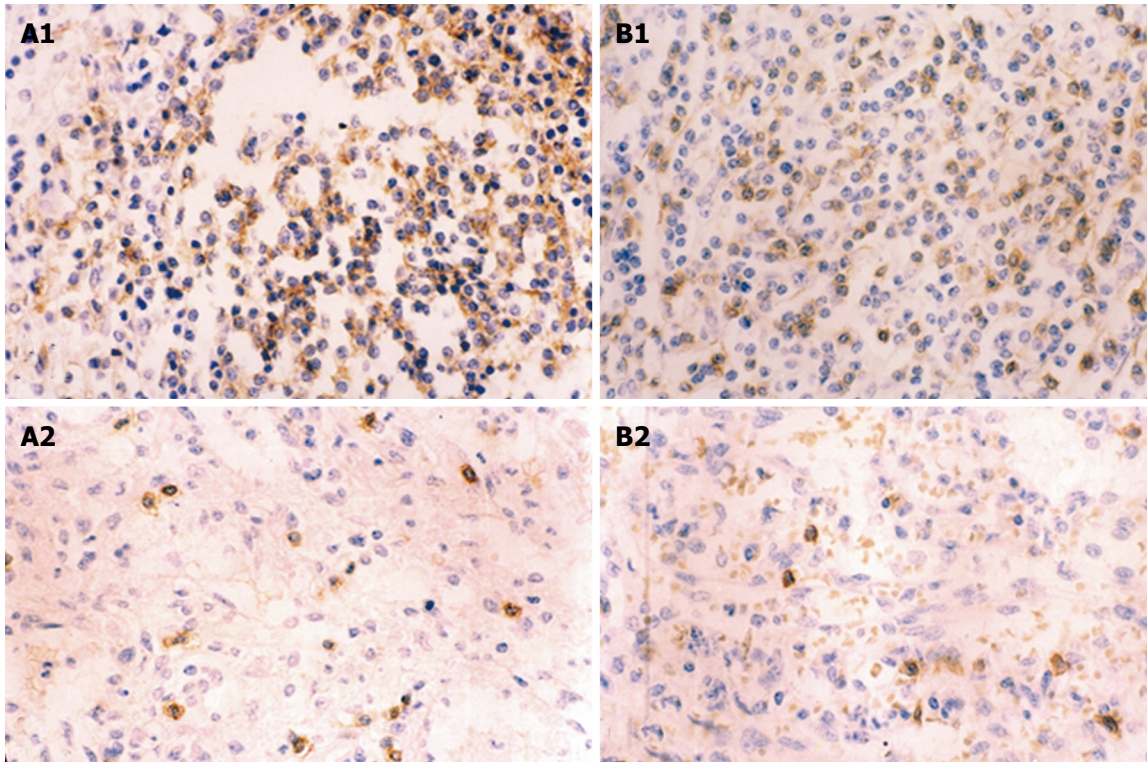


Figure 3 CD20 immunostaining for density of B lymphocytes. Compared to the control group, the density of B lymphocytes in the PH group decreased significantly in the splenic corpuscle and RP. **A1**: Control group, splenic corpuscle; **A2**: Control group, RP; **B1**: PH group, splenic corpuscle; **B2**: PH group, RP ($\times 100$).

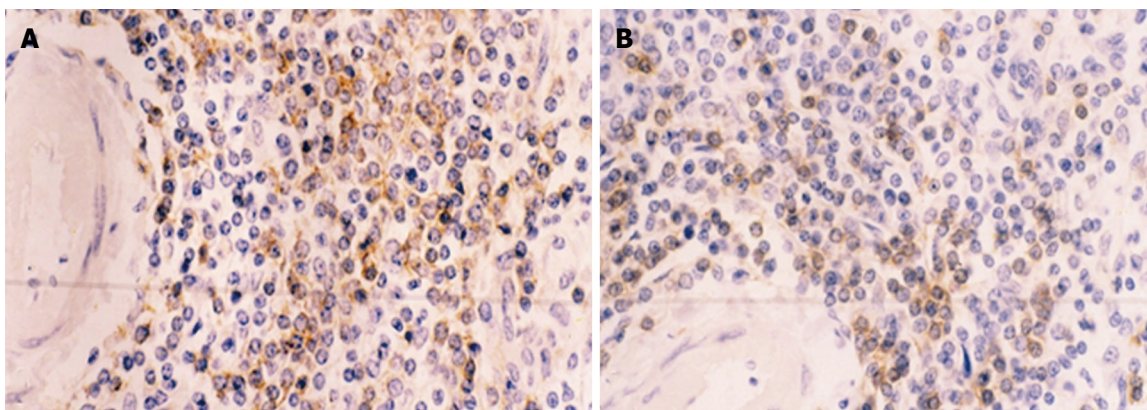


Figure 4 CD3 immunostaining for density of T lymphocytes. Compare to the control group, the density of T lymphocytes in the PH group decreased significantly in PALS. **A**: Control group; **B**: PH group ($\times 100$).

almost no differences between PH and normal spleens, while the lymphocyte density was significantly lower in the PH spleen. However, the lymphocyte density seen in a single microscopic field of view cannot represent the total lymphocyte count in the spleen. Therefore, in this

study, we purified and counted lymphocytes in the spleen, and then calculated lymphocyte count per gram of spleen tissue (relative quantity) and lymphocyte count in whole spleen (absolute quantity). This made the result more scientific and accurate. The results showed that although

the relative lymphocyte count per gram of spleen tissue was less in the PH spleen than that in normal spleen, as spleen weight increased greatly, the total lymphocyte count in whole spleen was significantly higher in the PH spleen than that in the normal spleen.

In addition, the proliferation of T and B lymphocytes is known as a response to stimulation induced by antigen or mitogens. The proliferative function is one of the important indices of lymphocyte immune function. Shi *et al* have reported that the expression of proliferating cell nuclear antigen (PCNA) in PH spleen is strongly positive in the lymphocyte aggregation area, which indirectly reflects the high proliferation status of lymphocytes^[21]. PCNA is isolated as a protein with elevated levels during S-phase of the cell cycle. Its expression level may be a marker of the S-phase and represent the proliferative function of cells^[22]. However, PCNA immunohistochemical staining cannot precisely distinguish S-phase and non-S-phase PCNA-positive cells under a light microscope^[23]. The result has limited reliability in reflecting lymphocyte proliferation. Furthermore, sample fixation, immunostaining, and other laboratory procedures have certain influences on the demonstration of PCNA. While cellular multiplication induced by Con A is commonly used to detect T lymphocyte immunity *in vitro*, LPS-induced activation of B cells and subsequent immunoglobulin synthesis reflect B-lymphocyte immunity^[24]. Therefore, the proliferative function of T and B lymphocytes was evaluated by MTT assay after being stimulated with LPS and Con A, respectively. The proliferative function of lymphocytes was also significantly higher in PH spleen than in the normal control group.

The total count and proliferative function of splenic lymphocytes increased in PH spleen. A possible reason is that long-term contact between noxious substances, such as endotoxin and hepatitis virus, and spleen tissue has promoted activation and hyperplasia of lymphocytes in the spleen^[16,25]. Also, this contact has enhanced its function to maximize the elimination of toxins in the body and maintain body balance^[26-29]. From this perspective, PH spleen has not completely lost immune function but does have some disorder. However, the immunological mechanism of the spleen is quite complicated. Hence, to confirm whether PH spleen has normal immune function^[30] and to achieve precise evaluation of the immune function of the PH spleen, further research should be conducted.

COMMENTS

Background

A better understanding of the function of the spleen has been gained recently, owing to in-depth studies on its structure, cellular function, secretion and innervation. It is generally accepted the spleen is an important part of the regulatory network between the immune, nervous and endocrine systems. The spleen has many more functions besides blood filtering and storage, hematogenesis, and immunization, and its immune function has characteristics of both "two-way" and "phase". Present knowledge about the immune function of the spleen in patients with PH is still incomplete; it is unclear whether preservation of splenic tissue with splenomegaly is beneficial to patients with PH and cirrhosis. Lymphocytes play a key role in the immune function of the spleen. Studies on splenic lymphocytes will be helpful for precise evaluation of spleen function, especially in a pathological state.

Research frontiers

It has previously been reported that phagocytosis of macrophages is enhanced

in the PH spleen, but there is little compelling experimental evidence on the distribution, count, and function of lymphocytes in the PH spleen.

Innovations and breakthroughs

This study proved the total quantity and the proliferative function of lymphocytes were increased. It suggests that the PH spleen may still have immune function, although perhaps with some disorder.

Applications

Although this was an initial study on the changes in lymphocytes in the spleen in PH, it may offer new evidence for complete evaluation of the immune function of the PH spleen.

Peer review

The authors investigated changes in the number and proliferative function of splenic lymphocytes in patients with PH. This was a very interesting study.

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Analysis of the human Atox 1 homologue in Wilson patients

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Author contributions: Simon I and Schaefer M contributed equally to this article. Simon I carried out the genetic tests and prepared part of the manuscript; Schaefer M has designed and coordinated the study, and prepared and revised the manuscript, and was active in acquisition of patients; Reichert J carried out some of the mutational analysis, data bank searches and statistical analysis and given technical assistance. Stremmel W improved the final manuscript and was active in acquisition of patients.

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Abstract

AIM: To analyze the metallochaperone antioxidant-1 (Atox1) gene sequence in Wilson disease patients.

METHODS: Mutation analysis of the four exons of the Atox1 gene including the intron- exon boundaries was performed in 63 Wilson disease patients by direct sequencing.

RESULTS: From 63 selected patients no mutations were identified after the entire coding region including the intron- exon boundaries of Atox1 were sequenced. One known polymorphism within the Atox1 gene (5'UTR -99 T>C) in 31 (49%) of the Wilson patients as well as one previously undescribed variation (5'UTR -68 C>T) in 2 of the Wilson patients could be detected. Statistical analyses revealed that the existence of a variation within the Atox1- gene showed a tendency towards an earlier onset of the disease.

CONCLUSION: Based on the data of this study, no major role can be attributed to Atox1 in the pathophysiology or clinical variation of Wilson disease.

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Key words: Antioxidant-1; Wilson disease; Wilson's disease protein; Mutation analysis

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INTRODUCTION

Copper is an essential trace element for prokaryotes and eukaryotes. It acts as a cofactor for a number of proteins such as cytochrome c oxidase, dopamine β -hydroxylase (production of catecholamines), superoxide dismutase (free radical detoxification), lysyl oxidase (cross-linking collagen and elastin) and ceruloplasmin^[1]. At the same time, excess or free copper is toxic to the cell.

The inherited disorders of copper metabolism Menkes and Wilson disease (WD) result from a disturbance of copper balance, resulting in either a deficiency (Menkes) or an accumulation of copper (WD) in the body^[2]. WD occurs in about one of 30 000 people^[3-5] and is characterized by the accumulation of copper primarily in the liver but also in the brain, kidney, cornea (Kayser-Fleischer-Rings), and spleen. WD is caused by a genetic defect in the ATP7B gene, located on chromosome 13q14.3^[6]. This gene encodes a polytopic membrane protein containing several motifs characteristic of P-type ATPases, highly expressed in the liver^[7]. Under steady-state conditions the gene product of ATP7B WNDP (Wilson's disease protein) resides in the trans-Golgi network (TGN)^[8] where it delivers copper to such secreted copper-dependent enzymes as ceruloplasmin. As the copper content of the hepatocytes increases, this ATPase moves from the TGN to a cytoplasmic vesicular compartment near the canalicular membrane. As copper is transported into this compartment, the intracellular copper concentration falls and the WNDP is recycled back to the TGN while copper is exported from the cell^[9].

There are more than 230 mutations in ATP7B^[10] accounting for Wilson Disease, and no mutation is predominant^[11]. Patients present, typically between the ages of 5 and 40 years, with quite various hepatic (40%), neurological (40%), psychiatric (14%-18%) or other symptoms^[12,13]. Although the specific type of mutation might have in part influence on disease severity, even patients with identical mutations show high clinical variability regarding the age of onset, signs and syndromes, ceruloplasmin levels, hepatic copper levels and presence of Kayser-Fleischer-rings^[14,15]. The following factors are

known to influence the disease: There are certain sex-specific differences: female patients for example show a higher prevalence of acute liver failure than male patients. Schiefermeier *et al* reported that an APO E ε3/3 genotype delays the onset of signs and symptoms^[16]. Merle *et al* described influence on onset of symptoms of Wilson disease depending on prion protein status^[17]. It has been proposed that genes influencing human copper metabolism might modify the clinical picture caused by a mutated Wilson disease gene.

As noted above copper is not free within the cell^[18]. The trafficking of copper from donor and acceptor proteins is mediated by a unique class of proteins termed copper chaperones^[19,20] that were first identified in the yeast *Saccharomyces cerevisiae*. The yeast ATX1p encodes a small cytosolic copper-binding protein that binds copper via the copper-binding MxCxC motif and delivers this metal to CCC2^[21] - the yeast homologue of WNDP - for subsequent transport into the secretory pathway and incorporation into the ceruloplasmin homologue FET3^[22]. The human homologue Antioxidant-1 (Atox1) is an 8 kDa cytosolic protein that contains a single copy of the highly conserved MxCxC motif^[23] in the amino terminus that is repeated 6-fold in WNDP. This metallochaperone interacts directly with the Wilson ATPase^[2] and can regulate its copper occupancy^[24]. By modulating the amount of copper bound to the protein Atox1 can regulate the intracellular localization^[25], the posttranslational modification^[26,27] and the enzymatic activity of WNDP^[24].

MATERIALS AND METHODS

Patients

The Genomic DNA of 63 WD patients was sequenced, which include 42 female and 21 male patients. The average age was 34 years (19-56). Twenty eight (44%) patients presented primarily with hepatic symptoms (including elevated liver enzyme test, ascites, liver cirrhosis, and acute liver failure), 20 (32%) patients primarily with neurological symptoms, 7 (11%) patient with both, 8 (13%) patients were diagnosed preclinically by family screening.

In all patients the definitive diagnosis of WD was established either by DNA analysis or by typical clinical and laboratory constellations [reduced copper levels (< 10 μmol/L) and ceruloplasmin levels (< 0.2 g/L) in the serum, raised free plasma copper levels, increased urinary copper excretion (≥ 2 μmol/24 h), detection of Kayser-Fleischer-rings, elevated hepatic copper concentrations (> 250 μg/g dry tissue)] or both. The ATP7B gene has been sequenced in most patients in part so far in cooperation with Professor Ferenci, Department of Gastroenterology and Hepatology, Vienna, including the analysis of the H1096Q mutation status in all patients and sequencing of exons 8, 13, 14, 15 and 18 in most patients not homozygous for H1096Q. In 36 patients two mutations could be detected within the Wilson disease gene. In 16 patients one mutation and in 11 patients no mutations could be detected within the Wilson gene so far.

Methods

Genomic DNA was isolated from peripheral EDTA-

Table 1 Oligonucleotide primers used to amplify Atox1

Exon	Sequence	Fragment size
Exon 1-Forward primer	5'-GGAGTGGGAGGGGC CTCCGGGACC-3'	273 bp
Exon 1-Reverse primer	5'-GTAAGCTAGGGGAC AACAGCGCTC-3'	
Exon 2 - Forward primer	5'-GCACTGTGTGGGGG TCACTCTACAG-3'	320 bp
Exon 2-Reverse primer	5'-GTGAGGATTAATG ATGTGATTCAC-3'	
Exon 3-Forward primer	5'-GAACTCTTCTTGCT GTAACGGGAG-3'	344 bp
Exon 3-Reverse primer	5'-GAGGGCTCTCCCGC TCCACTCAAG-3'	
Exon 4-Forward primer	5'-TGCAATGTCGCTAT GTCCACACCA-3'	326 bp
Exon 4-Reverse primer	5'-GATCACACAGCAA AGAATCAGAATC-3'	

blood using the QIAamp® DNA Blood mini Kit (Quiagen, Hilden/Germany) according to the manufacturer's instructions.

The four exons of the gene including the intron-exon boundaries were amplified by PCR, using the Oligonucleotide primers shown in Table 1, designed from the published cDNA sequence of the human homologue of Atox1 gene^[23].

The 50 μL volume of each PCR reaction contained 100 ng of template DNA, 500 μmol/L of each oligonucleotide primer, 200 μmol/L each dNTP, 2.5 units of Stratagene Pfu-DNA Polymerase (Qiagen, Hilden, Germany) in 10 × QIAGEN PCR Buffer (Qiagen, Hilden).

PCR reactions were performed in a Progene FPROG050 cycler (Techne, Cambridge/ UK) starting with the initial denaturation of the DNA at 95°C for 10 min; followed by 45 cycles (exon 1, 3, 4)/40 cycles (exon 2) of: 45 s denaturation at 95°C, 45 s annealing at 60°C (Exon 1)/56.5°C (Exon 2)/50°C (Exon 3 + Exon 4) and 1 min extension at 72°C, then with a final extension of 72°C for 10 min.

PCR products were purified with the MinElute™ Purification Kit (Quiagen, Hilden/Germany). The sequencing reaction was performed by SEQLAB (Sequence Laboratories Göttingen, Göttingen/Germany).

Sequences obtained by sample sequencing were compared with <http://www.ncbi.nlm.nih.gov/BLAST>.

Statistical analyses

Statistical analyses were performed with the SPSS, version 13.0 (Statistical Package for the Social Science, SPSS Inc., Chicago, IL, USA.). $P < 0.05$ was taken as significant.

RESULTS

We analysed Atox1 in 63 WD patients, diagnosed either by DNA analysis or by typical clinical constellations.

There could be no alterations in the Atox1 coding exon sequence or splice junction sequence be detected in any of these individuals.

Direct sequencing of the Atox1 gene within the 5'-UTR region located before exon 1 of the 63 Wilson disease

Table 2 Atox1 gene analysis in Wilson disease patients

Mutations in the ATP7B-gene	No. of patients	Distribution of the variations detected in the <i>Atox1</i> -gene among the <i>ATP7B</i> -genotypes				No variation within the sequence
		5'UTR -99 T>C		5'UTR -68 C>T		
		Heterozygous	Homozygous	Heterozygous	Homozygous	
H1096Q/H1096Q	22	6	4	-	-	12
R1041W/R1041W	2				-	2
G12810N/G12810N	1				-	1
G1266R/G1266R	1	1			-	
G710A/G710A	1					1
K844k-fs/K844k-fs	1					1
W779X/W779X	1		1			
D765N/Y741X	2		1			1
3400 Del C/2299InsC	1					1
3400 Del C/H1096Q	1	1				
3400 Del C/G982V	1	1				
3400 Del C/W779X	1					1
H1096Q/M769V	1	1				
m.n.d./m.n.d.	11	7		2		4
H1096Q/m.n.d.	8	3	1			4
W 778 X/m.n.d.	1	1				
W779X/m.n.d.	2		1			1
D765N/m.n.d.	2					2
G710S/m.n.d.	1		1			
M769V/m.n.d.	1					1
2299 InsC/m.n.d.	1	1				
Total	63	22	9	2	0	32

m.n.d.: Mutation not detected.

patients examined revealed one known polymorphism within the Atox1 gene in 31 (49%) of the Wilson patients (Table 2). Thirty one of 63 (49%) Wilson patients had detectable Atox1 gene changes, with the heterozygous T/C at 5'UTR -99 being the most common with 22 (35%) patients.

In 2 Wilson patients the previously undescribed 5'UTR -68 C>T (heterozygote) genetic variation could be detected.

Seven of the 11 patients where no Wilson disease gene mutation could be found so far and 8 of the 16 patients with a single identified Wilson disease gene mutation showed one of the variations in Atox1.

5'UTR -99 T>C

This polymorphism 5'UTR -99 T>C (Figure 1A) is already described in the database (NCBI Sequence Viewer: NM_004045: "dbSNP: 1549921"). 35% (22 patients) showed this substitution of the base T by C heterozygous, the other nine patients (14%) were homozygous for this variation.

Among these 22 patients with the heterozygous variation 7 persons presented with primarily hepatic symptoms, 7 patients with primarily neurological symptoms, and 3 patients with hepatic and neurological symptoms. Five were presymptomatic at diagnosis.

The nine patients with homozygous variation are composed of 3 patients with primarily hepatic symptoms, 3 persons with primarily neurological symptoms, 1 patient with hepatic and neurological symptoms, and 2 patients diagnosed preclinically by family screening (Figure 1B).

The heterozygote variation could also be found in one healthy control person, homozygote variation could be found in two persons without WD. The healthy control person, [ALT 24 U/L, AST 31 U/L (-35), copper

21 µmol/L (12-35), ceruloplasmin 0.27 g/L (0.2-0.6)] and two persons had no signs of Wilson disease (based on clinical and basic laboratory testing). They had normal levels of serum ceruloplasmin, copper and liver function tests.

5'UTR -68 C>T (heterozygot)

This additional variation of the 5'UTR could be found in two patients. One of them presented with the hepatic presentation, the other one had a mixed presentation (Figure 2).

The detected nucleotide changes in the 5'UTR region of the Atox1 gene did not have significant association with or influence on the average age of initial manifestation of Wilson disease (Table 3). Statistical analyses revealed that the existence of a variation within the Atox1- gene showed no significance towards an earlier onset of the disease (Table 4).

DISCUSSION

When sequencing the exons of the Wilson disease gene in clinically proven Wilson disease patients, only a single or even no mutation can be detected in 5%-10% of all patients depending on the population examined and the analyzing laboratory. In our study, a single Wilson disease gene mutation could only be identified in 16 patients and no Wilson disease gene mutation could be found in 11 patients so far. However complete sequencing is still under way in most of these patients.

Sixty three patients were analyzed as potential candidates of an Atox1 caused Wilson disease like disease. 31 out of these 63 patients had variations within the Atox1 gene. In terms of clinical presentation (age of onset, hepatic/

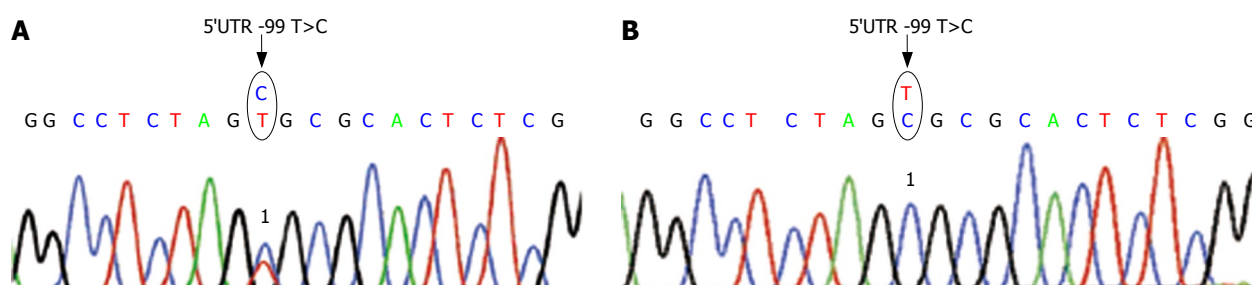


Figure 1 Sequence analysis of genomic DNA of the *Atox1*- gene 5'UTR -99T>C site. **A:** Heterozygote in this site. ¹Heterozygote substitution of the base T by C on position -99 in front of the beginning of the start codon located in exon 1; **B:** Homozygote in this site. ¹Homozygote substitution of the base T by C on position -99 in front of the beginning of the start codon located in exon 1.

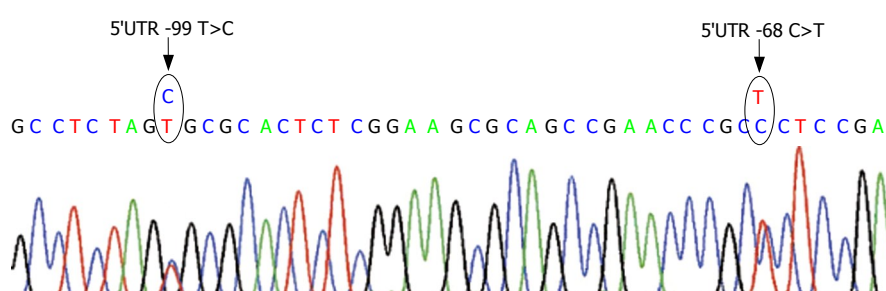


Figure 2 Localization of the 5'UTR -68 C>T gene variation in relation to the 5'UTR -99 T>C variation.

Table 3 Analysis of average ages of initial manifestation with regard to the variations detected in the *Atox1* gene

<i>Atox1</i> -variation	Average age of initial manifestation	<i>n</i> of patients	Standard deviation
No variation within the sequence	23.68	31	12.993
Variation within the sequence detected	18.88	25	9.909
Total	21.54	56	11.863

neurologic onset), laboratory tests (serum ceruloplasmin, serum copper, 24 h urinary copper excretion) and clinical course (improvements, drug reactions, side effects, initial neurological worsening) there were no significant differences between the 31 WD patients with *Atox1* changes compared to the remaining 32 patients without *Atox1* changes. As these sequence changes were in the 5'UTL region leaving the translated regions as well as the splicing sites and the classical translation initiation complex site of the gene intact, it seemed unlikely, that these detected changes could cause a Wilson disease like disease by themselves or be able to significantly influence the clinical course of Wilson disease. On the other hand, the highly conserved region of the translated *Atox1* gene in humans can be evidence for a vital role of *Atox1* protein in human metabolism or embryonic development. This also might explain the still unknown phenotype of *Atox1*-mutation associated diseases^[2], even though different roles and regulatory factors for *Atox1* in human metabolism are emerging out of recent studies^[28-31]. One might speculate about a Menkes disease like phenotype resulting from a complete disruption of both functional alleles of *Atox1* as suggested by *Atox1* knockout mice data^[32].

The association between the *Atox1* variations and the

Table 4 Statistical data for Analysis of age of initial manifestation

Model	Not standardized coefficients		Standardized coefficients		
	B	Standard error	Beta	T	Significance
ATP7B	4.042	1.970	0.272	2.052	0.045
<i>Atox1</i>	-5.312	3.079	-0.225	-1.725	0.091

changes in the age of onset was weak in this study similar to data published before^[2].

Taken together, *Atox1* associated modification of Wilson disease or Menkes disease are still not seen so far and the absence of mutations in the coding regions of the *Atox1* gene speak for an essential role of wild type *Atox1* in human metabolism.

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COMMENTS

Background

Cytoplasmic copper has to be transferred into the cellular excretory pathway by copper transport pumps. In patients with the copper storage disease "Wilson disease" the copper transporter ATP7B is defective. This transmembranous ATPase receives cytoplasmic copper from the copper chaperone antioxidant-1 (*Atox1*).

Research frontiers

Patients with Wilson disease show a wide variation in their clinical presentation.

Proteins interacting with the ATP7B copper transporter, such as Atox1, COMMD1 or chemical such as Platinum-complexes are one important area in explaining this phenomenon.

Related publications

Lutsenko S, LeShane ES, Shinde U. Biochemical basis of regulation of human copper transporting ATPases. *Arch Biochem Biophys* 2007; 463: 134-148 [PMID: 17562324]; Singleton C, Le Brun NE. Atx-1 like chaperones and their cognate P-type ATPases: copper-binding and transfer. *Biometals* 2007; 20: 275-289 [PMID: 17225061]; Merle U, Schaefer M, Ferenci P, Stremmel W. Clinical presentation, diagnosis and long-term outcome of Wilson's disease: a cohort study. *Gut* 2007; 56: 115-120, 314 [PMID: 16709660].

Innovations and breakthroughs

Proper ATP7B function is regulated by cytoplasmic factors (e.g. COMMD1, Atox1), cytoskeletal factors (e.g. dynactin subunit p62) and by hormones (e.g. prolactin, oestrogens). ATP7B and its sister protein ATP7A (Menkes protein) are regulated by tissue specific factors and by hormones and can be present simultaneously within one cell.

Applications

Research on modification factors of Wilson disease are aimed to identify protective factors within the clinical course of Wilson disease and providing them to affected patients.

Terminology

Atox1: antioxidant-1, small cytoplasmic protein with copper binding sides; ATP7B: copper transporting ATPase encoded by the Wilson disease gene; Wilson disease: autosomal recessive copper storage disease due to malfunction of ATP7B.

Peer review

This paper was well designed and analysed in large-scale patients. Although the results are in part negative, they are important to the scientific community.

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RAPID COMMUNICATION

Primary gastric mucosa associated lymphoid tissue lymphoma: Clinical data predicted treatment outcome

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CONCLUSION: Using univariate analysis, predictive factors for overall survival were international prognostic index (IPI) score, hemoglobin level, erythrocyte sedimentation rate (ESR), and platelet numbers ($P < 0.005$). In addition to this, Cox proportion hazard model differentiate IPI score, ESR, and platelets as predictors of survival.

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Key words: MALT lymphoma; Prognostic factors; Clinical features; Treatment

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Abstract

AIM: To determine clinical characteristics and treatment outcome of gastric lymphoma after chemotherapy and immuno-chemotherapy.

METHODS: Thirty four patients with primary gastric mucosa associated lymphoid tissue (MALT) lymphoma (Ann Arbor stages I to IV) were enrolled. All had upper gastric endoscopy, abdominal ultrasonography, CT and *H pylori* status assessment (histology and serology). After anti-*H pylori* treatment and initial chemotherapy, patients were re-examined every 4 mo.

RESULTS: Histological regression of the lymphoma was complete in 22/34 (64.7%) and partial in 9 (26.5%) patients. Median follow up time for these 31 responders was 60 mo (range 48-120). No regression was noted in 3 patients. Among the 25 (73.5%) *H pylori* positive patients, the eradication rate was 100%.

INTRODUCTION

Gastric low grade B cell lymphomas arising from mucosa associated lymphoid tissue (MALT) are the most frequent lymphomas among those located in the primary digestive tract. Approximately 40% of all non-Hodgkin's lymphomas occur in extranodal locations. The majority is of the diffuse large B cell type, but extranodal marginal zone B cell lymphoma of MALT is the commonest extranodal small B cell non-Hodgkin's lymphoma. Approximately 37% of extranodal lymphomas occur in the gastrointestinal tract and esophagus. The most common site is the stomach (23% of extranodal lymphomas) followed by the small intestine (7.5%) and the colorectum (5.5%). The initiating step in the pathogenesis of MALT lymphoma at all sites is the acquisition of organized lymphoid tissue. This will have the characteristic features of MALT with a germinal centre, mantle and marginal zone, plasma cell differentiation, and an associated T cell component. B cells may infiltrate epithelial structures, if present, to mimic a lymphoepithelium similar to that

seen in native MALT in Peyer's patches^[1]. The B cells of MALT lymphoma share the immunophenotype of reactive marginal zone B cells (CD20+, CD21+, CD35+, IgM+, and IgD-)^[2]. The analysis of the rearranged variable region of the immunoglobulin heavy chain gene by means of polymerase chain reaction (PCR) shows a monoclonal proliferation of neoplastic B cells^[3].

Gastric MALT lymphoma seems to develop along 2 major molecular pathways that emerge from the oncogenic inflammatory milieu of the stomach, one dependent on the presence of t (11; 18) and the other associated with a methylator-prone phenotype (CIMP). Four disparate chromosomal translocations are associated with extranodal MALT lymphoma, including t (11; 18) (q21; q21), t (1; 14) (p22; q32), t (14; 18) (q32; q21), and t (3; 14) (p14; q32)^[4]. They most frequently affect the *MALT1* gene, including both the t (11; 18) (q21; q21) and t (14; 18) (q32; q21), and interestingly, are mutually exclusive cytogenetic events. However, 3 of the translocations appear to involve a common pathogenic mechanism, leading to constitutive activation of NF- κ B signaling. It has been reported that lymphomas responding to *H pylori* therapy are t (11; 18) negative^[5], thus this translocation may be associated with more advanced disease. In the majority of cases in the stomach (but not all) the stimulus for acquisition of MALT is *H pylori* infection. Indeed, *H pylori* provides the antigenic stimulus which is mediated by mucosal T cells for sustaining growth of gastric MALT lymphoma. *H pylori* tumor cells show somatic hypermutation in the immunoglobulin genes that are characteristic of antigen selection^[6]. MALT lymphoma cells resemble memory B cells still responsive to differentiation signals, such as CD40 costimulation and cytokines produced by antigen-stimulated helper T cells, and dependent for their growth on stimulation by *H pylori*-specific T cells. Thus, *H pylori* stimulation of lymphoma B cells is not direct, but occurs through tumor-infiltrating T cells, involving both CD40 and CD40L costimulatory molecules. The surface immunoglobulin on gastric MALT lymphoma B cells does not recognize *H pylori*, but instead recognizes various autoantigens, suggesting that malignant cells are transformed from autoreactive B cells. Initially, MALT lymphoma is sustained by *H pylori*-induced T cell help, remains localized, and subsequently regresses^[7]. Since the first cases of gastric lymphoma (GL) regression after such eradication were reported in 1993, various remission rates of 41%-100% have been published for several low grade GL series^[8].

In contrast to primary gastric NHL, secondary involvement of the GI tract by nodal NHL, which occurs in 20% to 60% of newly diagnosed cases, reflects disseminated disease that necessitates systemic treatment strategies^[9,10]. Studies analyzing the incidence of secondary gastric NHL revealed a great discrepancy between the frequencies of GI involvement diagnosed before treatment as opposed to postmortem findings. The aim of this study was, therefore, to determine, in consecutive patients with gastric MALT lymphoma, the predictive factors of lymphoma regression in concordance with the course of the disease.

MATERIALS AND METHODS

Patients and diagnostic criteria

From July, 1997 to September, 2003, thirty four patients with primary gastric MALT lymphomas were enrolled in the study. Informed consent was obtained from all patients and the study was approved by the local Ethics Scientific Committee.

All patients underwent baseline endoscopy and gastric mucosal biopsies of the stomach. Lymphomas were staged clinically by the Ann Arbor system modified by Musshoff and Schmidt-Vollmer and by Rohatiner^[11,12]. Staging procedures included recording of the patients' physical examination, ileocolonoscopy, together with upper gastrointestinal tract endoscopy, small bowel and chest radiography, abdominal computed tomography (CT) scan, Waldeyer's ring examination with endoscopy and biopsies or CT scan, and bone marrow biopsy.

The histopathological diagnosis was based on the REAL classification. Histological specimens were stained with hematoxylin eosin (HE) and Giemsa (Figure 1). Immunohistochemical analyses (Dako, Glostrup, Denmark) were performed on all paraffin embedded biopsies, and MALT lymphoma cells, like marginal zone B lymphocytes, had phenotypic profile: CD20+, CD21+, CD35+, IgM+, CD5-, CD10-, IgD-, BCL-10+ (Figure 2).

All patients had HIV negative status (detected by ELISA testing-Axym, Abbott, USA, and Vitros, Ortho USA). *H pylori* was systematically looked for using the Giemsa stain method. Also, for culture, tissue biopsies were grounded, plated on three media (two selective and one non-selective), and incubated in a microaerobic atmosphere at 37°C for 7-10 d. *H pylori* was identified on the basis of positive oxidase, catalase, and urease tests.

Treatment

All patients with gastric MALT lymphoma received poly- or monochemotherapy (Cyclophosphamide, Doxorubicine, Vincristine, Prednisolone-CHOP; Chlorambucil, Vincristine, Procarbazine, Prednisolone-LOPP; or Chlorambucil) or combined immuno-chemotherapy (Rituximab-CHOP) as the first line treatment. Four patients were operated (subtotal gastrectomy). Patients who were *H pylori* positive, received triple anti-*H pylori* treatment combined with chemotherapy or immuno-chemotherapy as first line management.

Statistical analysis

Overall survival was calculated from the date of diagnosis until death or last follow-up. The actuarial survival curves were estimated using the Kaplan-Meier method. Student *t*-test was used to evaluate the difference in the values of clinical parameters. The log-rank test evaluated association between overall survival (OS) and clinical characteristics. The use of the Cox proportional hazards model determined independent prognostic factors which influenced OS, while logistic regression detected prognostic factors overall survival significant for outcome.

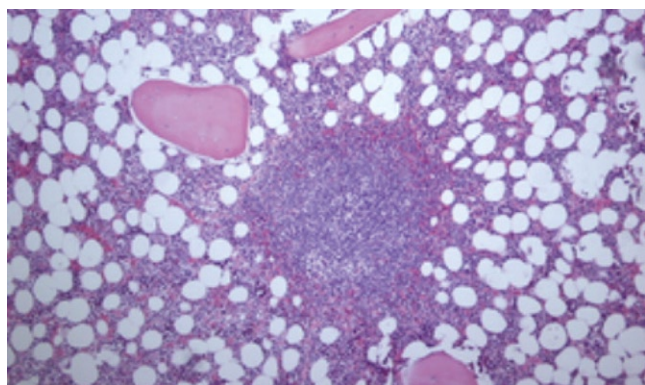


Figure 1 Bone marrow pathohistology in gastric MALT lymphoma patients (HE, × 100). Paratrabecular nodal lymphoid BM infiltration with neoplastic cells.

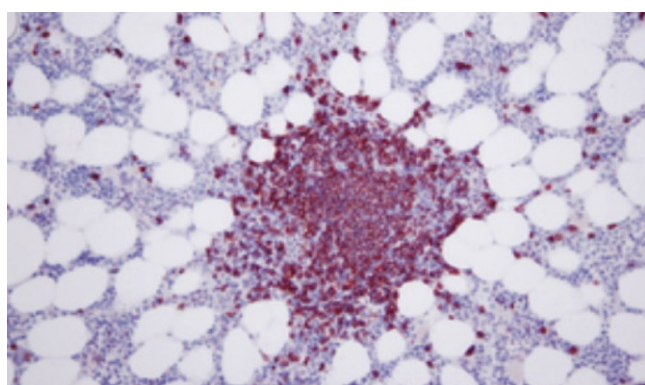


Figure 2 Immunohistochemistry of CD20 antigen expression in bone marrow biopsy (× 200). Nodal lymphoid BM infiltration with CD20+ in lymphoma cells.

RESULTS

A total of 34 (18 male, 16 female, median age 62 years, range 34-82 years) patients with gastric MALT lymphoma were included in the study. Median follow up was 72 mo (range 48-120). Accordingly to clinical stage (Table 1), all patients were treated with chemotherapy (23/34 i.e. 67.6%), monochemotherapy only in patients with IE2 clinical stage, or immunochemotherapy (11/34 i.e. 34.4%). The reason for giving chemotherapy or immunochemotherapy to all patients as first line treatment was the BCL-10 positivity of all patients with clinical stage I + II. There were no differences in outcome of patients between the groups with mono and polychemotherapy. Also, all patients who were *H. pylori* positive (25/34 i.e. 73.5%) received on d 0, the same anti-*H. pylori* treatment for 14 d: 40 mg Omeprazole twice daily, 1 g amoxicillin twice daily, and 500 mg Clarithromycin twice daily. The first endoscopy follow up examination was performed 30 d after the end of treatment to check both the effectiveness of *H. pylori* eradication, as assessed by histology, tissue culture, and PCR, and the absence of macroscopic tumor progression. Those patients with persistent *H. pylori* were tested for susceptibility to Metronidazole and given a second line treatment. Patients with stable and progressive disease, and one patient with partial remission were operated (subtotal gastrectomy)

Table 1 Modified Ann-Arbor clinical staging system

Clinical stage		Number of patients (n)
I E1	Mucosa + submucosa	0
I E2	Muscularis propria + subserosa	8
II E1	Perigastric lymph nodes	2
II E2	Regional lymph nodes	8
III E	Lymph nodes on both sides of the diaphragm	4
IV E	Visceral metastases or second extranodal site	12

Table 2 Clinical data and response to treatment

Clinical characteristics	n (%)
Dominant site of gastric lesion	
Fundus	10 (29.4)
Corpus	18 (53.0)
Antrum	4 (17.6)
Anemia (Hb < 110 g/L)	21 (61.7)
Thrombocytopenia (PLT < 100 × 10 ⁹ /L)	9 (26.5)
Elevated ESR (> 10/1 h)	16 (47.1)
Bone marrow infiltration	8 (23.5)
Constitutional "B" symptoms	28 (82.4)
Answer to therapy	
Complete remission	22 (64.7)
Partial remission	9 (26.5)
Stable disease	1 (2.9)
Progressive disease	2 (5.9)

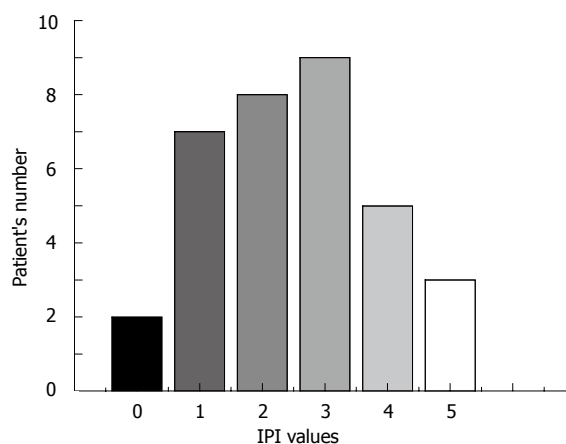


Figure 3 Distribution of IPI values in gastric MALT lymphoma patients. IPI score was a highly significant ($P < 0.01$) prognostic factor for overall survival of patients.

after restaging 4 mo after the starting initial systemic therapy.

Various clinical characteristics of patients with MALT lymphoma, and data relating to therapy response are summarized in Table 2. Distribution of IPI value showed that the most number of patients, nine, had an IPI score of 3 (Figure 3).

Patients had high remission rate to first-line chemotherapy or immunochemotherapy combined with anti-*H. pylori* treatment. Remission was obtained in 91.2%, CR in 64.7% and PR in 26.5% of patients. After the end of the follow up period 28 (82.4%) patients were still alive. The cause of death in all patients was disease progression.

The actuarial survival curve estimated high survival rate

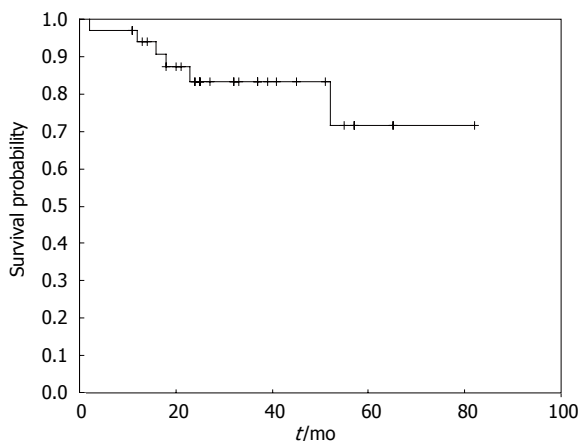


Figure 4 Kaplan-Meier curve of cumulative survival probability in gastric MALT lymphoma patients. Cumulative survival probability was high in the following period with low rate of death.

(83%) in the first 12 mo of the following period (Figure 4).

The use of the Cox proportional hazard model determined negative independent prognostic predictive factors for overall survival (OS): high IPI score, elevated ESR (erythrocyte sedimentation rate) and low platelets ($\chi^2 = 13.397$, $df = 3$, $P = 0.0039$).

Multivariate logistic regression showed the significance of elevated CS and low value of hemoglobin as independent prognostic factors that had negative influence for the outcome (dead or alive) of the disease ($df = 1$, $P = 0.0049$), whilst the elevated ESR had negative impact for achieving CR ($df = 1$, $P = 0.0107$).

DISCUSSION

We have described the clinical characteristics of primary gastric MALT lymphoma in 34 patients. Gastric MALT lymphoma offers a paradigm of infection-associated malignant disease. Most of them are multifocal and involve the antrum or distal body, but may occur in any part of the stomach^[13].

Classically, MALT lymphomas reveal a histological triad composed of reactive lymphoid follicles, diffuse infiltration of small irregular lymphocytes (centrocytes), and lymphoepithelial lesions (LELs). The histological features closely simulate those of Peyer's patches^[14].

The lymphoma infiltrates, around reactive follicles in the region corresponding to the marginal zone, were found to be spreading diffusely into the surrounding mucosa. The tumor cells typically resemble reactive marginal zone B cells, having moderate amounts of pale clear cytoplasm. However, the cytological appearance in individual cases was varied. A characteristic feature of MALT lymphomas is the presence of LELs formed by the invasion of glands by neoplastic cells.

We have focused on the patients treated with chemotherapy (67.6%) or immuno-chemotherapy (34.4%) as first line treatment, combined with triple way therapy against *H pylori* in *Helicobacter* positive patients. The reason for this approach was the fact that all patients in I and II CS expressed BCL-10, and also had constitutional "B"

symptoms, so we identified them as high risk patients. In about 25% of cases, resistance of gastric MALT lymphoma to *H pylori* eradication seems to be caused by chromosomal translocation involving the BCL-10 locus, such as t (1; 14) (p22; q32) and t (1;2) (p22; p12)^[15]. They are typically found at advanced stages and are unlikely to respond to *H pylori* eradication. GL bearing these translocations can be detected immunohistochemically by strong BCL-10 nuclear expression^[16].

In concordance with this is fact that patients who have t (11; 18) do not respond to anti-*H pylori* treatment. This translocation was described in 1989, and occurs specifically in MALT lymphoma, but not in other non-Hodgkin's lymphomas, including its absence in cases of nodal and splenic marginal zone lymphoma.

The translocation is absent from *H pylori* gastritis and other premalignant diseases^[17]. The t (1; 14) (p22; q32) and t (1; 2) (p22; p12) were described in MALT lymphoma in 1990 and 2000, respectively^[18,19]. They have been reported exclusively in MALT lymphoma and represent approximately 3% of gastric MALT lymphomas^[20].

The translocation t (11; 18) seems to favorably influence the response to *H pylori* eradication whereas its expression and the presence of BCL-10 mutations appear to be associated with failure to respond to antibiotics and with a more aggressive disease behavior^[21,22].

Under normal circumstances, signaling through the antigen receptor facilitates the interaction of BCL-10 and MALT1, which synergize to activate NF- κ B. BCL-10 forms a complex with MALT1 and the 2 molecules synergize in the activation of NF- κ B. In cases with t (11; 18) (q32; q21), the API2-MALT1 fusion protein activates the NF- κ B pathway through self-oligomerization. MALT lymphomas with these translocations show strong to moderate nuclear BCL-10 expression, and give anti-apoptotic signal to neoplastic lymphoid cells^[20-22].

There is some evidence that a few factors may influence a better chance of response. One factor may be limited extension of the disease, affecting only superficial layers of the stomach, low grade histopathology with low proportion of blast cells and clusters of blast cells^[23].

Other factors may be the specific gastric localization (with improved chances for gastric distal localization). The favorable response to *H pylori* eradication is rather exceptional and does not mean that all patients with localized (stage I) high grade gastric MALT lymphoma should be treated exclusively with eradication treatment. Rather it suggests that after eradication therapy as the initial treatment in patients with limited superficial disease, no infiltration of deeper layers of the gastric wall, and limited areas of high grade lymphoma, and most of all, when these patients can be closely monitored, chemotherapy may be postponed until follow up indicates whether or not further treatment is necessary. A prospective study of larger numbers of such patients may help to detect the factors that may predict a good response to *H pylori* eradication to avoid the use of an unnecessary and toxic treatment such as chemotherapy in patients that may already have been cured^[24,25].

In our group of patients, we have a large proportion

with advanced CS, III and IV (16 i.e. 47%), but the diagnosis was clear that it was primary gastric lymphoma. Obvious differences between primary and secondary gastric lymphoma became evident with regard to tumor localization and growth pattern. Secondary gastric NHL was seen more frequently as multifocal disease involving the gastric fundus and duodenum; both sites are rarely affected in primary gastric NHL like in our patients. In contrast to secondary gastric NHL, unifocal growth pattern is the most important endoscopic finding in primary gastric NHL. A unifocal growth pattern facilitates local radical treatment strategies, such as surgery, which may be associated with prolonged remission and a more favorable prognosis^[10,26].

As the part of diagnostic procedures, we used abdominal CT in concordance to modified Ann Arbor staging system. According to CS and findings that none of the patients had CS IE1 and these with CS IE2, CS IIE1 and more advanced staged were BCL-10+ with "B" symptoms, we made decision to aggressively treat them with or without anti-*H. pylori* treatment (in dependence of *H. pylori* status). The most recent studies presented the role of endoscopic ultrasound (EUS) as procedure that should be included as part of staging for the diagnosis and treatment of gastric MALT lymphoma. The presence or absence of deep submucosal invasion as assessed by EUS was the most critical factor for pretreatment assessment. Therefore, EUS has a much higher sensitivity in distinguishing stage IE from stage IIE1 lymphoma, compared with CT scan. Furthermore, with analysis of the response data according to depth of infiltration, the overall response rate was highest for mucosa stage IE1 and then decreased with the depth of gastric wall infiltration^[23,27-29].

Among all examined prognostic factors, the IPI score was the most powerful prognostic factor for OS, together with ESR and platelet number in the Cox hazard regression model ($P = 0.0039$). The distribution of IPI score showed that only two patients had IPI = 0, and the highest number of patients (9) had IPI of intermediate-high risk (IPI = 3). About one quarter of patients had thrombocytopenia, due to BM infiltration, which correlated with shorter survival. The standard biochemistry parameter ESR was elevated in one half of patients, and the acute phase reactant had prognostic influence on survival. In addition, elevated ESR had a negative influence on achieving CR by using multivariate logistic regression. Anemia, as a result of erythropoiesis suppression in malignant disease and due to BM infiltration, had a negative influence on disease outcome as well as high Ann Arbor modified clinical stage. Our findings are in agreement with prognostic factor analysis in described literature data^[29,30].

In contrary to persistence of disease, the basic goal would be prevention of MALT lymphoma appearance. Even though, *H. pylori* is a tumor-inducing pathogen, it does not induce malignant diseases in the vast majority of infected hosts. Therefore, vaccination strategies must acknowledge this co-evolution of *H. pylori* with its human host in which many of the host-pathogen interactions that occur could prove potentially beneficial^[31].

In conclusion, these data show that adequate patient selection for treatment option offers them a good chance

for long-term survival. Using novel diagnostic procedures, such as EUS, differentiate low risk patient candidates for only antibiotic approach and avoids an aggressive treatment option. As a final point, murine models using infection with several gastric *Helicobacter* species provided a distinctive experimental method to examine the progression of the MALT lymphoma from early immune responses to fully developed malignant disease. These systems offer the model of gastric lymphoma development through a series of molecularly events. Analysis of these steps using gene expression profiling gave some insights into the mechanisms involved in the lymphogenesis and can be prove in large prospective studies^[32].

COMMENTS

Background

Gastric low grade B cell lymphomas arising from mucosa associated lymphoid tissue (MALT) are the most frequent lymphomas among those located in the primary digestive tract. The translocation t (11; 18) seems to favorably influence the response to *H. pylori* eradication whereas its expression and the presence of BCL-10 mutations appear to be associated with failure to respond to antibiotics and with a more aggressive disease behavior. In about 25% of cases, resistance of gastric MALT lymphoma to *Helicobacter pylori* eradication seems to be caused by chromosomal translocation involving the BCL-10 locus, such as t (1; 14) (p22; q32) and t (1; 2) (p22; p12). They are typically found at advanced stages and are unlikely to respond to *H. pylori* eradication. Besides, few factors may influence a better chance of response. One factor may be limited extension of the disease, distal gastric localization, affecting only superficial layers of the stomach, low grade histopathology with low proportion of blast cells and clusters of blast cells.

Research frontiers

The favorable response to *H. pylori* eradication is rather exceptional and does not mean that all patients with localized (stage I) high grade gastric MALT lymphoma should be treated exclusively with eradication treatment. Rather it suggests that after eradication therapy as the initial treatment in patients with limited superficial disease, no infiltration of deeper layers of the gastric wall, and limited areas of high grade lymphoma, and most of all, when these patients can be closely monitored, chemotherapy may be postponed until follow up indicates whether or not further treatment is necessary.

Innovations and breakthroughs

We have focused on the patients treated with chemotherapy (67.6%) or immuno-chemotherapy (34.4%) as first line treatment, combined with triple way therapy against *H. pylori* in *Helicobacter* positive patients. The reason for this approach was the fact that all patients in I and II CS expressed BCL-10, and also they had constitutional "B" symptoms, so we identified them as high risk patients. Patients had high remission rate (91.2%) to first-line chemotherapy or immuno-chemotherapy combined with anti-*H. pylori* treatment. Among all examined prognostic factors, the IPI score was a powerful prognostic factor for OS, together with ESR and platelet number in the Cox hazard regression model ($P = 0.0039$).

Applications

These data show that adequate patient selection for treatment option offers them good chance for long-term survival.

Peer review

The manuscript is an interesting, generally well written series of gastric MALTOMA patients, reflecting on the impact of clinical factors upon outcome with current treatment.

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RAPID COMMUNICATION

Radiotherapy for 65 patients with advanced unresectable hepatocellular carcinoma

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Abstract

AIM: To evaluate the efficacy of radiotherapy (RT) in patients with advanced unresectable hepatocellular carcinoma (HCC).

METHODS: A total of 65 patients were treated with RT in the Korea University Medical Center. The median age of the patients was 60 years, and 86.2% were men. 18.5% and 81.5% of the patients were diagnosed as TNM stage III and IV-A, respectively. Treatment response was assessed 4 mo after initiation of RT. Tumor regression rate 1 mo after initiation of RT (TRR_{1m}) was also assessed. Duration of survival was calculated from the initiation of RT.

RESULTS: The objective treatment response was 56.9%. The 12 mo survival rate was 34.7%. Predictive factors for survival were Child-Pugh grade, α -fetoprotein level and treatment response. An objective response was achieved more frequently in patients with TRR_{1m} \geq 20% than in those with TRR_{1m} < 20% ($P < 0.001$).

CONCLUSION: RT is effective in treating advanced HCC with a tumor response rate of 56.9%.

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Key words: Hepatocellular carcinoma; Radiotherapy; Treatment response; Survival

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INTRODUCTION

In Korea, hepatocellular carcinoma (HCC) accounts for 83% of primary liver cancer, which is the third most common cancer and the third leading cause of cancer-related death^[1,2]. Although surgical resection is considered to be the treatment of choice for long-term control of HCC, this treatment is considered at diagnosis in less than 20% of HCC patients due to disease extent or a hepatic function that is inadequate for resection^[3-5].

Although percutaneous ablation therapy, such as percutaneous ethanol injection or radiofrequency ablation, could be the best treatment in patients who are not suitable for resection, this treatment is limited to early-stage HCC^[6]. Transarterial chemoembolization (TACE) is used for patients with unresectable HCC who are also ineligible for percutaneous ablation^[6]. However, because complete tumor necrosis is rare with TACE, repeated treatments are often needed. Additionally, TACE-induced vascular injury can limit further TACE^[7-10]. Finally, patients with portal vein thrombosis or extensive tumor burden are poor candidates for TACE, because their tumors are frequently associated with arterio-portal shunts and TACE-related liver damage. For these advanced HCCs, hepatic arterial infusion of chemotherapy (HAI) has yielded promising results in several recent studies^[11-13], but the benefit of HAI is still controversial.

Radiotherapy (RT) for the treatment of HCC has been attempted over the last four decades, but the results have been unsatisfactory because the doses were too low to be adequately tumoricidal^[14-16]. Recently, however, several studies have suggested local, high-dose RT is well tolerated and leads to a favorable treatment response in patients

with unresectable HCC^[17-20]. Therefore, this study was performed to evaluate the treatment responses of RT and survival in patients who underwent RT for unresectable HCC.

MATERIALS AND METHODS

Patients

This study was performed with patients who underwent RT for unresectable advanced HCC without distant metastases. Between July 2003 and June 2006, 80 patients with unresectable HCC underwent local RT to the liver at the Korea University Medical Center. Fifteen of these patients were excluded due to the presence of distant metastases prior to RT.

Diagnosis of HCC was based on either the identification of hypervascular masses by two imaging studies or by one imaging study combined with a serum alpha-fetoprotein (AFP) level > 400 ng/mL. If the vascular profile by dynamic imaging was not characteristic of HCC and the AFP was less than 400 ng/mL a biopsy was performed^[21]. Unresectability was determined using accepted surgical criteria^[3].

The baseline characteristics of the 65 patients are presented in Table 1. Fifty-six patients (86.2%) were male and 9 were female. The median age was 60 years (range, 42-83) years. Underlying liver diseases included chronic Hepatitis B virus (HBV) infection in 49 patients (75.4%), alcoholic liver cirrhosis in 13 patients (20%) and chronic Hepatitis C virus (HCV) infection in two patients (3.1%). In one patient (1.5%), co-infection with HBV and HCV was noted. Liver cirrhosis was present in 50 patients (76.9%). According to the Child-Pugh classification, 43 patients (66.2%) were classified as grade A and 22 patients (33.8%) were classified as grade B. Patients in class C were not included. Baseline tumor size was 10.8 ± 4.7 cm (median, 9.9 cm). In 31 patients (47.7%), the tumor size was larger than 10 cm. Based on the types of HCC described by Egge^[22], the most frequent tumor type was massive (58.5%), followed by multinodular (36.9%) and single nodular (4.6%). Prior to RT, portal vein thrombosis was observed in 45 patients (69.2%); this was confirmed by CT and/or angiogram. Among these 45 patients, thrombosis was observed in the main portal vein in 20 patients (30.8%), at the first branch level in 23 (35.4%), and at the second branch level in 2 (3.1%). The hepatic vein and bile duct were involved in 8 and 6 patients, respectively. No patients showed evidence of extrahepatic metastasis prior to RT. According to the TNM staging system of the Liver Cancer Study Group of Japan^[23], 53 patients (81.5%) fell into stage IV-A, and 12 (18.5%) fell into stage III.

Treatment

RT was performed as a primary treatment in 40 of the 65 patients (61.5%) due to an overly large tumor size in 20 patients (30.8%), portal vein thrombosis in 12 patients (18.5%), IVC thrombosis in 3 patients (4.6%), bile duct invasion in 3 patients (4.6%), and a massive portosystemic shunt around the tumor in 2 patients (3.1%). In the remaining 25 patients (38.5%), RT was performed as

Table 1 Baseline characteristics of the 65 patients who underwent radiotherapy for unresectable hepatocellular carcinoma

Characteristics	Number of patients (%)
Age (yr)	60 (42-83) ¹
< 60/≥ 60	35 (53.8)/30 (46.2)
Gender (Male/Female)	56 (86.2)/9 (13.8)
Underlying liver disease (viral/alcohol)	52 (80.0)/13 (20.0)
Liver cirrhosis	50 (76.9)
Ascites	24 (36.9)
Child-Pugh class (A/B)	43 (66.2)/22 (33.8)
Albumin (g/dL) ²	3.4 ± 0.5
Bilirubin (mg/dL) ²	1.2 ± 0.9
Alkaline phosphatase (IU/L) ²	151.8 ± 79.3
Platelet (10 ³ /mL) ²	156.3 ± 66.2
Prothrombin time (INR) ²	1.2 ± 0.2
Sodium (mEq/L) ²	137.7 ± 3.7
Creatinine (mg/dL) ²	1.0 ± 0.9
Tumor size (cm) ²	10.8 ± 4.7
< 10/≥ 10	34 (52.3)/31 (47.7)
Tumor type (SN/MN/massive)	3 (4.6)/24 (36.9)/38 (58.5)
Portal vein thrombosis	45 (69.2)
UICC stage (III/IV-A)	12 (18.5)/53 (81.5)
α-fetoprotein (IU/mL) ²	17454 ± 66005
> 400/≤ 400	28 (43.1)/37 (56.9)
Radiotherapy aim (primary/salvage)	40 (61.5)/25 (38.5)

¹Median (range); ²mean ± SD; INR: International normalized ratio; SN: Single nodular; MN: Multinodular.

a salvage treatment after ineffective TACE (21 patients, 32.3%) or vascular inaccessibility to the feeding vessel of the HCC (4 patients, 6.2%). External beam RT at a target dose of 61 Gy/34 fractions was planned, using 10 MV of X-rays. The RT strategy was devised using a CT-based 2-D planning system (CT Port, Toshiba, Tokyo, Japan). To account for respiratory-based liver motion, a 1-1.5 cm margin was added in the craniocaudal direction. The full 61-Gy irradiation dose was feasible in 55 of the 65 patients (84.6%).

During and after RT, TACE was also employed in 57 patients (87.7%; 2.9 ± 1.8 sessions; median, three sessions; range, 1-8 sessions). TACE was performed with an emulsion of doxorubicin at a dose of 10-30 mg and 4-12 mL of mixed solution of lipiodol and contrast agent. TACE was usually combined with embolization using gelfoam particles, except in cases with significant portal vein thrombosis. In 16 patients with portal vein invasion (24.6%), HAI with cisplatin and 5-FU was combined with or without TACE (3.3 ± 2.4 cycles; median, 2.5 cycles; range, 1-8 cycles).

Evaluation of treatment response

Tumor size was measured by computed tomography (CT) and was calculated as the longest diameter multiplied by the longest perpendicular diameter. CT scans were obtained before RT, 1 and 4 mo after the initiation of RT, and then every 2-3 mo. If a patient had multiple nodules, the extent of the tumor was determined by the sum of the extent of all tumors > 2 cm in diameter.

Treatment response was assessed at four months after initiation of RT. A complete response was defined as the complete disappearance of all clinical and radiographic tu-

mor evidence. A partial response was defined as more than a 50% decrease in tumor size from baseline. Stable disease was defined as less than a 50% decrease or a 25% increase in tumor size. The objective treatment response was calculated based on the complete and partial responses. Progressive disease was defined as a greater than 25% increase in extent of the tumor from the nadir extent of the tumor.

To evaluate the efficacy of using the early tumor response to predict the treatment response, the tumor regression rate at one month after initiation of RT (TRR_{1m}) was assessed using the following equation: TRR_{1m} = [(baseline tumor extent - tumor extent at one month after RT)/baseline tumor extent] × 100.

Adverse events were evaluated weekly during RT and one month following the treatment. Adverse hematologic events were evaluated by measuring hemoglobin, white blood cell (WBC) and platelet counts, while hepatic adverse events were evaluated by measuring serum bilirubin, albumin, aspartate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphatase (ALP) levels. Gastrointestinal (GI) bleeding included any bleeding from the esophagus, stomach, duodenum, or liver. All adverse events were graded according to Common Terminology Criteria for Adverse Events V3.0.

Statistical analysis

All calculations were performed using SPSS 10.0 software for Windows (SPSS, Chicago, IL). Quantitative variables were expressed as mean ± SD or medians. Differences in quantitative and qualitative variables were assessed using the Student's *t*-test and chi-square test, respectively. Logistic regression analysis was performed to evaluate predictive factors for tumor response. Survival and progression-free survival were assessed from the initiation of RT according to the Kaplan-Meier method. Differences between variables were assessed using the log-rank test. The Cox regression model was used to detect associations between survival and AFP status, tumor type, the location of tumor thrombi, stage and therapeutic models. For multivariate analysis, variables with *P* < 0.2 at univariate analysis were entered. Differences with *P* < 0.05 were considered to be statistically significant.

RESULTS

Tumor response

Fifty-five of 65 patients (84.6%) completed the RT schedule. Ten patients (15.4%) could not complete RT due to HCC aggravation or deterioration of liver function after RT. Interruption of RT was more frequent in Child-Pugh class B patients (7 of 20 patients, 35%) than in class A patients (3 of 45, 6.7%; *P* = 0.003). Among the 55 patients who completed RT, treatment response was evaluated 4 mo after the initiation of RT. None of our patients had completely responded at this point in the response evaluation, but 37 patients (67.3%) had partially responded. Seventeen patients (30.9%) had stable disease, and 1 (1.8%) had progressive disease. Therefore, the objective treatment response at four months was 67.3%.

Table 2 Baseline characteristics of the 65 patients, according to treatment response

	Pts without OTR (<i>n</i> = 28)	Pts with OTR (<i>n</i> = 37)	<i>P</i> value
Age (yr)	61 ± 10	58 ± 8	0.271
Gender (M:F)	22:6	34:3	0.124
Hepatitis B	21 (75%)	28 (75.7%)	0.950
Hepatitis C	2 (7.1%)	1 (2.7%)	0.573
Alcohol abuse	5 (17.9%)	8 (21.6%)	0.707
WBC (/mm ³)	5450 ± 1671	6079 ± 2229	0.216
Hemoglobin (g/dL)	11.4 ± 2.2	12.0 ± 1.8	0.207
Platelet (× 10 ³ /mm ³)	160 ± 738	154 ± 608	0.733
AST (IU/L)	111 ± 93	71 ± 53	0.032
ALT (IU/L)	62 ± 51	69 ± 90	0.686
ALP (IU/L)	175 ± 89	134 ± 67	0.038
Bilirubin (mg/dL)	1.47 ± 1.27	0.91 ± 0.49	0.035
Albumin (g/dL)	3.3 ± 0.4	3.5 ± 0.5	0.103
Prothrombin time, INR	1.16 ± 0.22	1.15 ± 0.14	0.796
Creatinine (mg/dL)	1.17 ± 1.31	0.92 ± 0.25	0.329
Liver cirrhosis	21 (75%)	29 (78.4%)	0.749
Child-Pugh grade A	14 (50%)	31 (83.8%)	0.003
Alpha-fetoprotein (ng/dL)	31474 ± 98160	6844 ± 15814	0.199
≥ 400 ng/dL	15 (53.6%)	22 (59.5%)	0.635
Tumor size (mm)	118 ± 37	99 ± 52	0.112
≥ 10 cm	18 (64.3%)	14 (37.8%)	0.035
Multiple tumor	24 (85.7%)	30 (81.1%)	0.622
Massive type	18 (64.3%)	20 (54.1%)	0.407
Main portal vein thrombosis	10 (35.7%)	10 (27%)	0.452
Tumor stage IV	23 (82.1%)	30 (81.1%)	0.913

OTR: Objective treatment response; WBC: White blood cell; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; ALP: Alkaline phosphatase; INR: International normalized ratio; Tumor stage: According to the TNM staging system of Liver Cancer Study Group of Japan.

However, if we label the 10 patients who did not complete RT as non-responders, then the partial response and stable disease rates decreased to 56.9% (37 of 65 patients) and 26.2% (17 of 65 patients), respectively.

Table 2 presents baseline characteristics according to treatment response. Logistic regression analysis was performed to evaluate predictive factors for an objective treatment response. Child-Pugh grade was the only independent predictive factor for an objective treatment response (Child-Pugh grade A *vs* B; OR, 5.167; 95% CI, 1.643-16.250; *P* = 0.005). Among the 45 patients with Child-Pugh grade A, 31 patients (68.9%) showed a partial response, as did 6 of the 20 grade B patients (30%, *P* = 0.003).

Time to progressive disease

During follow-up, 4 of the 37 patients showing a partial response (10.8%) and 3 of the 25 patients showing stable disease (12%) had progressive disease after a median of 6 (range, 3-9) mo. Among the 65 patients, time to progressive disease was 5 ± 3 mo after initiation of RT (median, 4 mo). Duration without progressive disease was longer in patients who met the objective treatment response (14.8 ± 1.4 mo) than in patients who did not (4.6 ± 0.4 mo, *P* < 0.001; Figure 1).

Survival

All enrolled patients were followed for 8 ± 6 (median,

6; range, 1-30) mo. During this period, 37 patients died. Fourteen patients died of hepatic failure, 13 died of HCC aggravation, 5 died of gastrointestinal bleeding, 2 died of tumor rupture, and 2 died of sepsis. The cumulative survival rates at 6, 12 and 18 mo were 61.5%, 34.7% and 27.0%, respectively. Patients who showed an objective treatment response (median survival, 346 d) survived longer than those who did not (median survival, 212 d; $P = 0.032$; Figure 2).

When multivariate Cox-regression analysis was performed with baseline characteristics, large tumor size (≥ 10 cm *vs* < 10 cm; OR, 2.416; 95% CI, 1.213-4.811; $P = 0.012$), Child-Pugh grade B *vs* A (OR, 4.094; 95% CI, 1.977-8.480; $P < 0.001$) and the presence of tumor thrombi in the main portal vein (OR, 2.315; 95% CI, 1.156-4.634; $P = 0.018$) were independent predictive factors for mortality. However, when multivariate analysis was performed after inclusion of the objective treatment response, Child-Pugh grade B *vs* A (OR, 3.706; 95% CI, 1.718-7.996; $P = 0.001$), high serum AFP level (OR, 2.459; 95% CI, 1.187-5.094; $P = 0.015$) and a failure to meet the objective treatment response (OR, 5.619; 95% CI, 2.475-12.760; $P < 0.001$) were independent prognostic factors for mortality (Table 3).

Tumor regression rate at 1 mo after RT initiation

TRR_{1m} was assessed in all 65 patients. TRR_{1m} was more than 20% in 41 patients (63.1%). Of the 41 patients with a TRR_{1m} $\geq 20\%$, 35 patients (85.4%) showed a partial response, while only 2 (8.3%) of the 24 patients with TRR_{1m} $< 20\%$ showed a partial response ($P < 0.001$; Figure 3). When logistic regression analysis was performed after inclusion of TRR_{1m} among the variables used to predict objective treatment response, Child-Pugh grade A (OR, 0.121; 95% CI, 0.019-0.784; $P = 0.027$) and TRR_{1m} $\geq 20\%$ (OR, 158.302; 95% CI, 14.032-1785.827; $P < 0.001$) were independent predictive factors.

Adverse events

Adverse events during and one month after RT are summarized in Table 4. Adverse hematologic events were identified in 78.5% of patients. Although most of them were mild and transient, grade 3 or 4 adverse events were noted in six patients (9.2%). Grade 3 or 4 hematologic adverse events were more frequent in patients who underwent RT combined with HAI (4 of 16 patients, 25%) than in patients with RT alone (2 of 49, 4.1%; $P = 0.012$). Adverse hepatic events were identified in 51.8% of the patients; the most common were hypoalbuminemia (33.8%) and hyperbilirubinemia (24.6%). Grade 3/4 adverse hepatic events developed in four patients (6.2%), with hyperbilirubinemia or elevation of AST in 1 (1.5%). Grade 3/4 adverse hepatic events were more frequent in patients with TRR_{1m} $< 20\%$ (4 of 24 patients, 16.7%) than in those with TRR_{1m} $\geq 20\%$ (1 of 41, 2.4%; $P = 0.038$). GI bleeding developed in five patients. The status of two patients with peptic ulcer disease and one patient with variceal bleeding was improved with medical or endoscopic treatment. However, two patients with hemobilia or HCC rupture expired after these events.

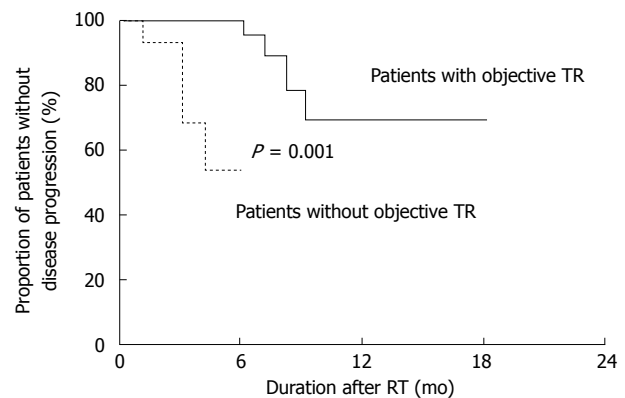


Figure 1 Duration without disease progression, according to treatment response. TR: Treatment response; RT: Radiotherapy.

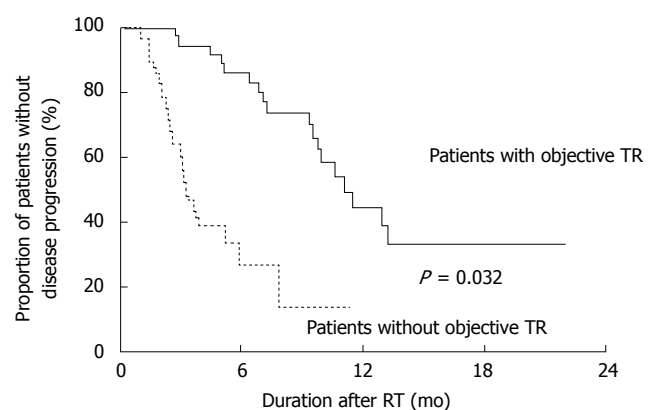


Figure 2 Overall survival of the 65 patients who underwent radiotherapy for advanced hepatocellular carcinoma according to treatment response. Patients with objective treatment responses (median survival, 346 d) survived longer than those without objective treatment responses (median survival, 212 d; $P = 0.032$). RT: Radiotherapy; TR: Treatment response.

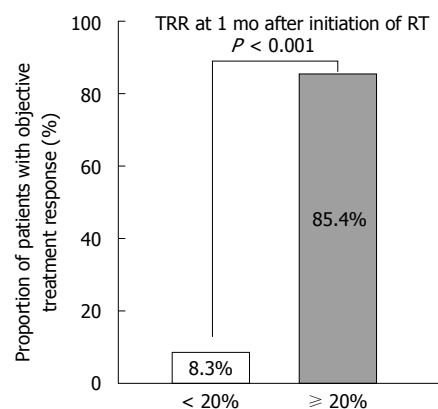


Figure 3 Proportion of patients who achieved an objective treatment response according to the tumor regression rate at one month after the initiation of radiotherapy. RT: Radiotherapy.

DISCUSSION

Recently, a number of reports have documented the effect of local RT on HCC^[17-20]. Although fractionation schemes were not identical to each other, local, high-dose RT alone or in combination with another modality such as

Table 3 Multivariate analysis of the mortality of patients who underwent radiotherapy for advanced hepatocellular carcinoma

		<i>P</i> value	β	Odds ratio	95% CI
Tumor size	0 ≤ 10 cm; 1 ≥ 10 cm	0.012	0.954	2.597	1.232-5.473
Child-Pugh grade	0 = Grade A; 1 = Grade B	0.001	1.336	3.802	1.687-8.568
Combined with TACE	0 = Yes; 1 = No	0.001	1.671	5.315	2.015-14.018
Objective treatment response	0 = Yes; 1 = No	0.006	1.194	3.300	1.414-7.699

Table 4 Adverse events in the 65 patients who underwent radiotherapy for unresectable hepatocellular carcinoma *n* (%)

	Grade					
	0	1	2	3	4	5
Hematologic	14 (21.5)	33 (50.8)	12 (18.5)	5 (7.7)	1 (1.5)	-
Hepatic	32 (49.2)	20 (30.8)	8 (12.3)	4 (6.2)	1 (1.5)	-
GI hemorrhage			1 (1.5)	2 (3.1)		2 (3.1)

TACE^[19,24], systemic chemotherapy^[25] or intra-arterial chemotherapy^[26,27] has achieved a substantial objective response. In this study, RT was performed with or without other treatment modalities and the objective response rate was 56.9%, which was somewhat lower than previous reported^[24,28-30]. However, when 10 patients (15.4%) who did not complete the whole RT schedule were excluded, the objective treatment response rate increased to 67.3%. We have no idea how the patients who could not complete RT were treated during these previous studies, because this was not reported. It is possible that all patients completed RT in the previous studies. However, a significant proportion of patients could not complete the RT schedule in the present study. Therefore, selection of appropriate patients for RT may be very important before RT initiation.

Child-Pugh grade was the only significant predictive factor for treatment response. This seems to be associated with the higher proportion of Child-Pugh grade B patients (35%) who could not complete the RT schedule compared with those with grade A (6.7%; *P* = 0.003). This speculation is supported by the fact that no variable was significantly associated with treatment response when the logistic analysis was performed on the 55 patients who completed RT (data was not shown). These results suggest that a circumspective decision was required in considering RT for patients with Child-Pugh grade B. Previously, tumor size was the one significant factor affecting treatment response^[29]. Similarly, a treatment response was more frequently seen in patients with a smaller HCC (23 of 32 patients, 69.7%) than in patients with a larger HCC (14 of 32 patients, 43.8%; *P* = 0.035). However, when multivariate analysis was performed, the significance disappeared.

Our results suggest RT may improve prognosis in patients who achieved an objective treatment response. RT appears to be associated with prolonged survival as well as prolonged suppression of HCC progression in patients who show an objective treatment response.

After the effects of other prognostic factors were corrected for, patients who achieved objective treatment responses survived longer than those who did not, as determined by multivariate analysis. In addition, time to progression was significantly longer in patients who met the objective treatment response than in patients who did not. However, several limitations should be discussed. First, 10 patients who could not complete the RT schedule were included in this analysis. However, even though these 10 patients were later excluded, patient survival still differed according to treatment response (*P* = 0.002; data not shown). Second, most patients were treated with not only radiotherapy, but also with TACE or HAI, and these combined treatments may affect patients' survival. To ideally assess the effect of RT on patient prognosis, RT should be the only treatment modality. However, considering the limitations of dose and field of RT, it seems unwise to use RT as the only treatment modality for advanced HCC.

In this study, the one-year survival rate was 34.7%, which was lower than in previous studies^[24,28-31]. It may be the patients enrolled in this study had more advanced disease than those in previous studies^[24,28-31]. In the present study, tumors were larger than 10 cm in 47.7% of patients, 69.2% of the cases had thrombi in portal vein, and 81.5% of the patients had stage IV-A disease. In addition, 10 patients (15.4%) who could not complete RT schedule were included in this study; none of these patients survived more than four months after initiation of RT. By contrast, most of the previous studies included patients who completed the RT schedule^[24,28-31], which may have led to the observed discrepancies with the present study.

In recent studies, PVT was the one prognostic factor for survival^[30,32]. Similarly, in this study, the presence of tumor thrombi in main portal vein as well as Child-Pugh grade and tumor size were independent prognostic factors for survival when multivariate analysis was performed with variables of baseline characteristics. However, when treatment response was included in the analysis, Child-Pugh grade, AFP level and treatment response were associated with survival. This result suggests that even if tumor thrombi are present in main portal vein before RT, RT may still improve survival when an objective treatment response is achieved.

In all of our patients, CT was performed at one month after initiation of RT. Tumor response at one month after initiation of RT was a useful predictor for RT response. In addition, grade 3 or 4 adverse hepatic events were more frequent in patients with TRR_{1m} < 20%. These results suggest if the mass does not decrease to 20% from baseline after one month of RT, interruption of RT can be considered due to the likelihood of a low objective treatment response rate and a high rate of severe adverse hepatic events.

In conclusion, RT was effective for the treatment of HCC with an objective tumor response rate of 56.9%; moreover, patients who met the objective treatment response survived longer than those who did not. Tumor regression at one month after the initiation of RT may be a useful predictor for RT response as well as severe adverse hepatic events.

COMMENTS

Background

Hepatocellular carcinoma (HCC) is one of the most common causes of cancer-related death in the world, especially in Asia. In a large proportion of patients, HCC is diagnosed at an advanced stage and, in this stage, widely performed treatment modalities including surgical resection, local ablation therapy and transarterial chemoembolization are not indicated. Recently, several reports have suggested high-dose radiotherapy (RT) could be an effective treatment option for advanced HCC.

Research frontiers

Most previous studies have included only patients who completed the RT schedule. However, according to our experience, some proportion of patients could not complete the whole RT schedule and their prognosis was usually very poor. Therefore, this might lead to a selection bias when analyzing the treatment response and survival of patients. In this study, all patients with HCC who were treated with RT for more than 1 mo during the study period were included. In addition, we evaluated the prognostic significance of early tumor response by follow-up CT at 1 month after the initiation of RT.

Innovations and breakthroughs

RT was effective in patients with advanced HCC with an objective tumor response rate of 56.9%. Early tumor response rate at 1 month after the initiation of RT was shown to be a good prognostic indicator for RT response. In addition, severe adverse events were more frequent in patients with poor early tumor response rates.

Applications

RT could be considered as a treatment option for patients with advanced HCC. If the tumor does not decrease to 20% from baseline after one month of RT, interruption of RT can be considered due to the likelihood of a low objective treatment response rate and a high rate of severe adverse hepatic events.

Peer review

This is an interesting article, which may offer new insights in the treatment of advanced unresectable HCC. The paper is well organized and the results are clearly described and commented.

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Is there correlation between pancreatic enzyme and radiological severity in acute pancreatitis?

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Abstract

AIM: To investigate the correlation between the changes of pancreatic enzyme, the biochemical markers and the clinical results according to the Balthazar computer tomography (CT) grade.

METHODS: Between July 2004 and July 2005, we reviewed the charts of 119 patients who were admitted to our hospital with acute pancreatitis.

RESULTS: Eighty-three patients (69.7%) were male, and the mean age of the patients was 57 ± 15.7 years. The biliary pancreatitis patients had an older mean age. Forty-nine patients (41.1%) had biliary pancreatitis and forty-six (38.6%) had alcoholic pancreatitis. Group 3 patients had a longer duration of pain (2.51 ± 1.16 vs 3.17 ± 1.30 vs 6.56 ± 6.13 , $P < 0.001$), a longer period of fasting (7.49 ± 4.65 vs 10.65 ± 5.54 vs 21.88 ± 13.81 , $P < 0.001$) and a longer hospital stay (9.17 ± 5.34 vs 14.63 ± 8.65 vs 24.47 ± 15.52 , $P < 0.001$) than the other groups. On the univariate analysis, the factors that affected the radiological grade were the leukocyte count at admission ($P = 0.048$), the hemoglobin ($P = 0.016$) and total bilirubin concentrations ($P = 0.023$), serum lipase ($P = 0.009$), the APACH II scores at admission ($P = 0.017$), the APACH II scores after 24 h ($P = 0.031$), the C-reactive protein (CRP) titer ($P = 0.0001$) and the follow up CRP titer ($P = 0.003$). But the CRP level ($P = 0.001$) and follow up CRP titer ($P = 0.004$) were only correlated with the radiological grade on multivariate analysis. According to the ROC curve, when we set the CRP cut off value at 83 mg/L, the likelihood

ratio for a positive test was 3.84 and the likelihood ratio for a negative test was 0.26 in group 3.

CONCLUSION: In conclusion, our study suggests that the CRP with the radiological severity may be used to estimate the severity of acute pancreatitis.

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Key words: Acute pancreatitis; Computed tomography; C-reactive protein

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Kim YS, Lee BS, Kim SH, Seong JK, Jeong HY, Lee HY. Is there correlation between pancreatic enzyme and radiological severity in acute pancreatitis? *World J Gastroenterol* 2008; 14(15): 2401-2405 Available from: URL: <http://www.wjgnet.com/1007-9327/14/2401.asp> DOI: <http://dx.doi.org/10.3748/wjg.14.2401>

INTRODUCTION

Acute pancreatitis is major cause of acute abdominal pain, and it is caused by alcohol ingestion, biliary stone, idiopathic causes and therapeutic endoscopy^[1,2]. The clinical presentation of the acute pancreatitis is variable. Most of these patients recover without specific complications, but some patients display severe complication such as pancreatic ascites and pancreatic necrosis; these patients show high mortality^[3,4].

The diagnostic markers (pancreatic enzymes such as amylase and lipase) and the risk factors that affect the clinical outcome are not well established by the previous clinical studies^[5-7]. However, we have reservations about any correlation of various diagnostic markers, including pancreatic enzymes and the clinical features, to the outcome of acute pancreatitis. Further, the recent development of radiological diagnostic instruments allows for a more accurate diagnosis and easier follow up^[8-10]. So in this study, we determined the correlation between the levels of pancreatic enzyme and the radiological severity of acute pancreatitis.

MATERIALS AND METHODS

Between July 2004 and July 2005, we reviewed the re-

Table 1 Characteristics of each group (%)

	Total	G-1	G-2	G-3
Sex				
Male	83 (69.7)	8 (23.5)	30 (25.2)	25 (21.0)
Female	36 (30.3)	13 (10.9)	16 (13.4)	7 (6.0)
Mean age (yr)	55.7 ± 15.7	51.2 ± 15.3	62.4 ± 14.6	51.9 ± 14.8
Etiology				
Biliary	49	21 (42.9)	21 (42.9)	7 (14.2)
Alcoholic	46	8 (17.0)	19 (41.3)	19 (41.3)
Drug	3	2 (67.0)	0 (0.0)	1 (33.3)
Idiopathic	21	10 (47.6)	6 (28.6)	5 (23.8)
Radiologic grade		41 (34.3)	46 (38.6)	32(26.8)

G-1: Balthazar CT grade A, B; G-2: Balthazar CT grade C; G-3: Balthazar CT grade D, E.

cords of 119 patients who were admitted to Chung Nam National University Hospital with acute pancreatitis. The diagnosis of acute pancreatitis was based on typical symptoms, including acute abdominal pain and a serum amylase level that was three times higher than the normal limit. After diagnosis is established, computed tomography (CT) scanning was performed to determine the findings and grade of disease. We excluded cases which the CT scan was not performed. We reclassified the CT grade into three groups. Group 1 was CT grade A + B, group 2 was CT grade C and group 3 was CT grade D + E. Serum amylase and lipase levels, the equivalent series resistance (ESR), and C-reactive protein (CRP) were tested and measured at admission, and again at 24, 48 and 72 h after admission. Also, the Ranson score and APACHE II score were calculated at the same time. The above markers were also measured when the patients started their oral diet. Abdominal CT was performed weekly to evaluate the change of severity of pancreatitis before starting an oral diet. We evaluated the correlation of these various factors and the clinical severity of acute pancreatitis.

Statistical analysis

Each result was calculated as a mean value and standard error. We analyzed the data using the SPSS 13.0 for windows. Chi-square tests, Student *t*-test, One-Way ANOVA test and MANOVA tests were used. For the different scoring systems, the sensitivity, specificity and overall correctness of prediction, the positive and negative predictive values and the likelihood ratios of the positive and negative tests were determined. Each score value obtained from the different scoring systems was used to calculate the different true positive (sensitivity) and false positive (1-specificity) rates to create the ROC curves. A *P* value less than 0.05 was considered a statically significant result.

RESULTS

A total of 119 acute pancreatitis patients were included in this study. The characteristics of the patients are shown in Table 1. Eighty-three patients (69.7%) were male, and the mean age of the patients was 57 ± 15.7 years. The biliary pancreatitis patients mean age was older than that of the alcoholic pancreatitis. Forty-nine patients (41.1%) showed biliary pancreatitis and there were forty-six (38.6%)

Table 2 Serum pancreatic enzyme (at initial) according to etiology

	Amylase	Lipase	L/A ratio
Alcohol	614 ± 466.9	2227.2 ± 1988.8	4.3 ± 2.4
Biliary	1107.6 ± 1185.9	3561.6 ± 3023.8	3.9 ± 2.0
Drug	427 ± 273.5	1320.3 ± 811.7	3.1 ± 0.1
Idiopathic	893 ± 1026.9	2481.9 ± 2983.9	3.6 ± 2.5

Reference values: Total amylase 13-65 IU/L, lipase 0-200 IU/L.

alcoholic pancreatitis patients. Forty-six patients suffered with drug-associated pancreatitis and 21 patients suffered with idiopathic pancreatitis. Various autoantibodies were checked for rule out autoimmune pancreatitis for the idiopathic pancreatitis patients. According to radiological severity, there were 41 (34.5%) patients in-group 1, 46 (38.6%) patients were in-group 2 and 32 (26.8%) patients were in-group 3. The CT grade was severe in the alcoholic pancreatitis patients. The serum pancreatic enzyme level at admission was higher in the biliary pancreatitis patients than in the alcoholic pancreatitis patients, but statistical significance was not present (Table 2).

Early elevation of serum pancreatic enzyme showed a statically significant result for the severe pancreatitis group. In this group, 3 of the patients' initial serum lipase concentrations were higher than that of the other groups and this decreased more rapidly during 24 h (*P* = 0.008) after admission. The serum albumin level, BMI and Ranson score are known to be predictive risk factors for acute pancreatitis, but in our study, they did not correlate with the CT grade.

What factors were associated with radiological severity grade in our study? On univariate analysis, old age, the initial leukocyte count, the initial hemoglobin level, the initial APACH II score and the APACH II score after 24 h, the initial serum lipase concentration and initial CRP titer were all correlated with the CT grade (Table 3). But, the initial CRP level and follow up CRP titer were only correlated with the radiological grade on multivariate analysis (Table 4). Between the CRP, APACH II score and the Ranson score (Table 5), the CRP titer is more predictive and this diagnostic test result will raise the pre-test probability for prediction of the radiological severity. Group 3 patients had a longer duration of pain (2.51 ± 1.16 *vs* 3.17 ± 1.30 *vs* 6.56 ± 6.13, *P* < 0.001), a longer fasting period without an oral diet (7.49 ± 4.65 *vs* 10.65 ± 5.54 *vs* 21.88 ± 13.81, *P* < 0.001) and a longer hospital stay (9.17 ± 5.34 *vs* 14.63 ± 8.65 *vs* 24.47 ± 15.52, *P* < 0.001) than other groups. Also, the complication rate of pancreatitis was higher in-group 3 than in the other two groups (*P* < 0.005).

According to the ROC curve (Figure 1), when we set the CRP cut off value at 83 mg/L, the likelihood ratio for a positive CPR test was 3.84 and the likelihood ratio for a negative CPR test was 0.26 in group 3. This result showed good correlation with the radiological grade in the course of acute pancreatitis (Table 6).

DISCUSSION

Acute pancreatitis arises from a variety of causes. The

Table 3 Univariate analysis of predictive factors

	G-1	G-2	G-3	P-value
BMI	2.5 ± 0.7	2.3 ± 0.7	2.2 ± 0.6	0.16
Leukocytosis (/mm ³)	11249.0 ± 6964.3	10555.0 ± 4212.5	17700.6 ± 23913.3	0.048 ^a
Hemoglobin (g/dL)	13.7 ± 1.9	13.4 ± 2.1	14.8 ± 2.3	0.016 ^a
Platelet (10 ³ /mm ³)	223.3 ± 92.1	220.5 ± 61.2	232.0 ± 95.8	0.829
AST (IU/L)	233.2 ± 253.9	337.9 ± 645.8	176.3 ± 393.7	0.31
ALT (IU/L)	212.0 ± 221.8	201.5 ± 235.2	139.2 ± 237.9	0.368
Total bilirubin (mg/dL)	2.8 ± 2.2	2.2 ± 1.9	1.5 ± 1.1	0.023 ^a
Albumin (g/dL)	3.9 ± 0.4	3.8 ± 0.4	3.8 ± 0.6	0.51
S-amylase (IU/L)	768.3 ± 926.4	762.7 ± 517.3	1243.1 ± 1531.7	0.079
S-lipase (IU/L)	1944.2 ± 1560.4	2820.3 ± 2521.4	3862.8 ± 3586.6	0.009 ^a
Ranson (initial)	1.7 ± 1.3	2.1 ± 1.2	1.8 ± 1.1	0.272
Ranson (24 h after)	0.7 ± 0.7	1.2 ± 1.0	0.9 ± 0.9	0.052
APACH- II (initial)	4.8 ± 4.3	7.5 ± 4.3	6.0 ± 4.5	0.017 ^a
APACH- II (24 h after)	3.5 ± 3.0	5.5 ± 3.8	4.7 ± 3.8	0.031 ^a
CRP (initial) (mg/dL)	4.5 ± 5.2	5.5 ± 4.5	11.9 ± 7.1	0.000 ^a
CRP (f/u)	2.4 ± 3.7	2.4 ± 2.5	6.6 ± 5.5	0.003 ^a

^aP < 0.05.

Table 4 Multivariate analysis of predictive factors

Factors	P value
Leukocytosis	0.29
Hemoglobin	0.07
Total bilirubin	0.31
S-lipase	0.44
APACH- II (initial)	0.65
APACH- II (24 h after)	0.47
CRP (initial)	0.001 ^a
CRP (f/u)	0.004 ^a

^aP < 0.05.

process of this disease varies from mild to severe necrotic pancreatitis. Most of these patients recover through the conservative management^[11,12]. But, a few patients progress to pancreatic necrosis and multi-organ dysfunction. In this study, the mortality was very high and near 30%^[13].

There was no gold standard therapy for acute pancreatitis except for conservative management such as fasting and injection of analgesics. Therefore, prediction of severity has not yet been important for the treatment of acute pancreatitis. However, this will be an important task in the future for the preventive and treatment of complications because various drugs and therapy strategies are now being tried^[6].

We know that various methods have been used to predict the progress of acute pancreatitis, such as clinical evaluation (include multivariate analysis of the prognostic factors), radiological evaluation *via* abdominal

Table 5 Likelihood ratio for prognostic markers at two time intervals

Marker	Initial	24 h after
CRP	2.22/0.39	2.09/0.46
Ranson	1.22/0.71	1.09/0.87
APACH- II	1.23/0.70	1.01/1.00

Positive likelihood ratio/negative likelihood ratio; Positive likelihood ratio = sensitivity/(1-specificity); Negative likelihood ratio = (1-sensitivity)/specificity.

Table 6 CRP test

Cut-off value	8.3	5.8	2.7
Sensitivity	70.6%	73.5%	69.6%
Specificity	81.6%	68.4%	68.7%
Likelihood for positive test	3.84	2.33	2.22
Likelihood for negative test	0.26	0.44	0.45

CRP: C-reactive protein (reference value: 0-5 mg/L).

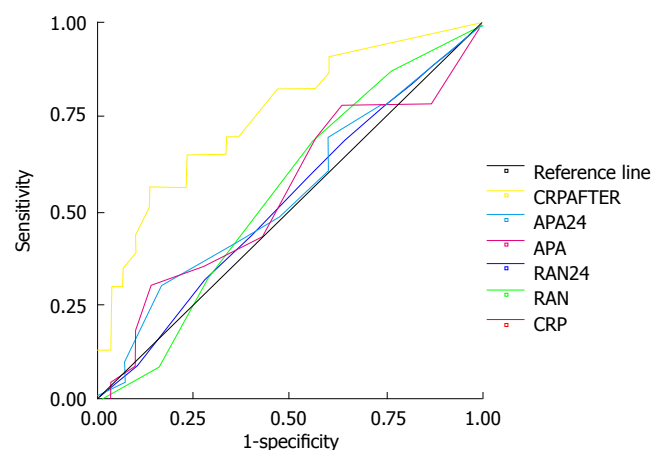


Figure 1 ROC curve predictive severe radiologic severity.

CT, and testing of various serological markers^[14-18]. The development of abdominal CT allows precise estimation of the severity of pancreas and retroperitoneal lesion. Furthermore, changes of parenchymal necrosis are now being evaluated using a dye that shows the changes of the microcirculation in the pancreas. Abdominal CT was recently used for determining the presence and diagnosis of complications in acute pancreatitis^[8,9,19]. The CT grade has shown good correlations with the clinical course and the prediction of mortality, so this is good method as compared to multivariate estimation of different factors^[17,20]. In our study, the CT grade was a more predictive method for the clinical course, that is, the higher the radiological grade, the longer the pain duration, the fasting duration and the hospital stay. But, those factors that represent the progress of acute pancreatitis have limitations.

We investigated the correlation between the changes of the pancreatic enzyme, the biochemical markers and the clinical results according to the Balthazar CT grade. We knew that acute pancreatitis was more frequent in six-

decade old males. The biliary pancreatitis patients were older than the alcoholic pancreatitis patients, but any radiological differences were not present. Most patients were of an alcoholic and biliary origin. We reported the difference of the pancreatic enzyme concentrations according to the etiology of pancreatitis. Our study showed that the serum amylase and lipase concentrations are higher in the biliary pancreatitis patients than the pancreatitis patients of an alcoholic origin. In another study, patients with severe biliary pancreatitis tended to have higher serum amylase levels at admission than the other groups^[21].

On the univariate analysis, the factors that affected the radiological grade were the leukocyte count at admission, the hemoglobin level, the total bilirubin concentration, serum lipase, CRP and the follow up CRP. The serum lipase concentration was used to diagnose acute pancreatitis due to its high specificity and sensitivity, but it cannot predict disease prognosis and severity. Also, in our study, the serum lipase concentration at admission was elevated in proportion to radiological severity, but it is not correctly correlated with the radiological severity on multivariate analysis. There were significant differences in each group for the APACH-II score at admission and also at 24 h. The estimation of severity through the Ranson criteria is not precise and is not an appropriate method because this method needs 48 h to complete and it has low specificity and sensitivity (77% and 75%, respectively). There was no significant correlation between the Ranson criteria and the radiological grade in our study.

The APACH-II score is the sum of the various physiologic parameters. This score has been used for evaluation of severe patients^[22-24]. It has high specificity and sensitivity for acute pancreatitis. In our study, groups 2 and 3 showed high sensitivity and specificity compared to group 1. But, the APACHE II score allows monitoring both the disease progression and the response to therapy, but the system is complex, difficult to perform and less accurate for identification of local complications^[25]. On our multivariate analysis, the complex APACHE II system was not correlated with the radiological grade when compared to the easily detectable CRP concentration, and the APACHE II system had a lower positive likelihood ratio and accuracy rate.

CRP is an acute stage protein that's synthesized in the liver. This parameter is usually used because it is simple and cheap^[26,27]. CRP is known to be a significant factor in the differential prognosis of acute pancreatitis^[5,28-30]. In this study, the CRP titer was only a predicative factor with good correlation to the radiological grade on multivariate analysis. Our results show statically significant differences at admission for prediction of severity. Also, changes of the CRP level during treatment reflect the prognosis of disease. When the cut-off value of CRP was 8.3 g/L, the positive predictive value was 38.4 mg/L in-group 3, and the negative predictive value was 0.26 in group 3. This single factor was the most precise and accurate for determining the radiological severity.

In conclusion, our study suggests that the CRP with the radiological severity may be used to estimate the severity of acute pancreatitis.

COMMENTS

Background

The diagnostic markers (pancreatic enzymes such as amylase and lipase) and the risk factors that affect the clinical outcome are not well established in acute pancreatitis. Abdominal computer tomography (CT) has recently been used for determining the presence and diagnosis of complications in acute pancreatitis patients.

Research frontiers

Previous studies haven't showed that clinical evaluation and biochemical marker predict severity in acute pancreatitis. The CT grade has shown good correlations with clinical course and the prediction of mortality.

Innovations and breakthroughs

C-reactive protein (CRP) and radiological severity have good correlation and may be used to estimate the severity of acute pancreatitis. CRP is easily measurable and is a simple method. CT is a good method which allows the prediction of clinical course and mortality, but it is expensive. Therefore, CRP is alternative method and clinically useful for prediction severity.

Peer review

This interesting study suggests that the CRP with the radiological severity may be used to estimate the severity of acute pancreatitis. The contents of the manuscript are reasonable, and this may be a useful method for prediction of clinical course and mortality, as the author's state.

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RAPID COMMUNICATION

Stronger inhibition of gastric acid secretion by lafutidine, a novel H₂ receptor antagonist, than by the proton pump inhibitor lansoprazole

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than by LPZ.

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Abstract

AIM: To compare the antisecretory activity and plasma drug concentrations of a single oral dose of 10 mg lafutidine, a novel H₂ receptor antagonist, with those of the proton pump inhibitor lansoprazole (LPZ) 30 mg.

METHODS: Ten volunteers without *H. pylori* infection participated in this crossover study comparing lafutidine 10 mg with LPZ 30 mg. Intragastric pH was monitored for 6 h in all participants, and blood samples were collected from four randomly selected individuals after single-dose administration of each drug.

RESULTS: The median intragastric pH was significantly higher in individuals who received lafutidine 10 mg than in those who received LPZ 30 mg 2, 3, 4, 5, and 6 h after administration. Maximal plasma drug concentration was reached more promptly with lafutidine 10 mg than with LPZ 30 mg.

CONCLUSION: In *H. pylori*-negative individuals, gastric acid secretion is more markedly inhibited by lafutidine

INTRODUCTION

Gastroesophageal reflux disease (GERD) commonly occurs in the western countries^[1,2], and its prevalence is now increasing in Japan^[3,4]. Recently, Ohara *et al*^[5] have shown that 42.2% of Japanese adults experience heartburn, similar to the rate of 42.4% reported in western studies^[1]. Gastric acid has an important role in the pathogenesis of GERD. Suppression of gastric acid secretion is the most common therapeutic approach, and more effective and promptly acting treatments are required.

Two types of potent gastric acid-suppressing agents, proton pump inhibitors (PPIs) and histamine H₂ receptor antagonists (H₂RAs), are widely used to treat GERD. PPIs such as lansoprazole (LPZ), rabeprazole, and omeprazole, the most potent acid inhibitors available, are often used for first-line treatment. Controlled studies have demonstrated that PPIs are far more effective than H₂RAs in patients with GERD^[5-9]. In the treatment of reflux esophagitis, H₂RAs have a number of disadvantages compared to PPIs, including shorter lasting efficacy and the development of tachyphylaxis, both limiting routine use^[10]. In contrast, PPIs are highly effective, and produce profound and sustained inhibition of gastric acid secretion, making these agents the mainstay of treatment for GERD.

GERD has a high rate of relapse. The rising use of PPI therapy on demand has raised issues regarding efficacy. Several studies have demonstrated that on demand therapy with PPIs provides an alternative to continuous treatment in patients with non-severe GERD^[11,12]. However, pH monitoring studies^[13-15] have shown that PPIs require 2 d to 3 d to inhibit acid secretion efficiently. In contrast, H₂RAs potently and promptly suppress gastric acid secretion^[16,17]. In this respect, H₂RAs might have advantage over PPIs, especially on the first day of treatment, i.e., when used on demand, for GERD. Furthermore, concerning the characteristics of GERD in Japan, it is important to distinguish the significant difference of acid secretion in Japanese patients when compared with that of western countries^[18,19]. Acid secretion among Japanese patients is lower compared to that of western population irrespectively of the status of *H. pylori* infection. Moreover, endoscopic studies^[3,4] have shown that GERD is mild in most Japanese patients. H₂RAs are thus sometimes used for the treatment of mild-to-moderate GERD in Japan.

Lafutidine is a newly synthesized H₂RA. Previous studies have shown that lafutidine promptly inhibits gastric acid secretion not only at night but also during the day^[16], in contrast to other conventional H₂RAs. Since patients with GERD often have symptoms during the day, we evaluated lafutidine in this study.

Few studies^[17] have examined the correlation between intragastric pH and blood drug concentrations in the early phase (1-6 h) after a single dose of H₂RAs or PPIs. The acid inhibitory activity of PPIs depends significantly on cytochrome P450 (CYP) 2C19 genotype, as well as on intrinsic pharmacokinetic and pharmacodynamic characteristics and dosing schemes^[20,21]. CYP2C19 genotypes were therefore determined for all participants in this study.

The major aim of this study was to compare the antisecretory activity of a single oral dose of 10 mg lafutidine (H₂RA) with that of a single dose of 30 mg LPZ (PPI). We also examined the correlation between intragastric pH and plasma drug concentrations during the early phase (1-6 h) after single-dose administration.

MATERIALS AND METHODS

Participants

Ten healthy male volunteers aged between 24 years and 48 years (mean, 28.7 years) and weighing 55 kg to 86 kg (mean, 68.6 kg) were included. Nobody of them had a history of gastrointestinal or hepatobiliary disease or of *H. pylori* eradication therapy. None were receiving regular medication. All volunteers gave written informed consent. The study protocol was approved by the ethical committee of Tohoku University Graduate School of Medicine.

Detection of *H. pylori* infection

H. pylori infection was diagnosed by the ¹³C-urea breath test^[22]. A total of 10 *H. pylori*-negative volunteers were invited and agreed to participate in this study.

Cytochrome P450 (CYP) 2C19 genotyping

After obtaining informed consent, a venous blood sample

was collected from all participants. DNA was extracted from the nuclei of venous white blood cells. Genetic mutations were analyzed by either the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method^[23] or by the TaqMan PCR amplification method (Applied Biosystems Japan, Chiba, Japan^[24]). On the basis of point mutations in exons 4 and 5, CYP2C19 gene status can be classified as homo-extensive metabolizer (homo-EM), hetero-extensive metabolizer (hetero-EM), or poor metabolizer (PM)^[25-27]. Homo-EM has wild type alleles (wt/wt) without any mutation in exons 5 or 4; PM has mutated alleles (m1/m2) with mutations in both exons 5 and 4 (m1/m2, m1/m1, or m2/m2); and hetero-EM has a mutated allele in either exon 5 or 4 (wt/m1 or wt/m2).

Study protocol

All subjects (homo-EM = 3, hetero-EM = 6, PM = 3) participated in an open-label crossover study with lafutidine 10 mg or LPZ 30 mg. They were randomly assigned to receive a single oral dose of lafutidine 10 mg tablets or LPZ 30 mg capsule at a fixed time. A washout period of at least 14 d intervened between the two study periods. Intragastric pH was monitored for 6 h after drug administration. To monitor intragastric pH, a pH electrode was inserted transnasally and positioned fluoroscopically in the gastric corpus, approximately 10 cm below the esophagogastric junction. Intragastric pH was measured at 10-second intervals by means of a portable pH meter attached to a glass pH electrode (Chemical Instrument, Tokyo, Japan). The pH electrode was calibrated before each recording, using standard buffers of pH 1.68, 4.01, and 6.86. The pH data were analyzed using a commercially available software (Chemical Instrument). No food was allowed, and 100 mL of tap water was allowed only when participants felt thirsty. All subjects were instructed to remain upright; normal daily activities were not restricted.

Sample collection and assay for lafutidine and LPZ plasma concentration

To study the correlation between intragastric pH and plasma drug concentrations, blood samples were randomly collected from four individuals (No. 3, 5, 6, 7) in heparinized tubes before and 0.25, 0.5, 1, 1.5, 2, 3, 4, and 6 h after drug administration. Blood samples were immediately centrifuged at 3000 r/min for 10 min. All samples were stored at -20°C until assay. Plasma LPZ levels were measured by high performance liquid chromatography/tandem mass spectrometry^[28,29]. This method requires only 20 µL of serum and is a simple procedure. Analytes and the internal standard (lansoprazole deuterium derivatives) were separated using a mobile phase of acetonitrile/1 mmol/L ammonium formate (140/60, mL/L) on a C18 analytical column and analyzed in the selected reaction-monitoring (SRM) mode. Detection limit was 500 fg/20 µL.

Plasma lafutidine concentrations were determined by high-performance liquid chromatography (HPLC), using 2-phenyl-1H-benzimidazol as an internal standard (IS). A 1 mL plasma sample added to 0.5 mL of 1 N NaOH and 0.05 mL of IS (20 µg/mL) was mixed with 5 mL of

Table 1 Characteristics of study participants

Subject	CYP2C19	Age	Height (cm)	Body weight (kg)	BMI
1	Homo-EM	23	171	70	23.9
2	Homo-EM	26	170	58	20.1
3	Homo-EM	28	169	60	21.0
4	Hetero-EM	22	165	60	22.0
5	Hetero-EM	28	170	80	27.6
6	Hetero-EM	40	172	75	25.3
7	Hetero-EM	44	181	82	25.0
8	PM	23	172	60	20.2
9	PM	23	175	55	18.0
10	PM	30	174	86	28.4

Homo-EM: Homo-extensive metabolizer; Hetero-EM: Hetero-extensive metabolizer; PM: Poor metabolizer.

n-hexane/dichloromethane (1:1). The mixture was shaken and then centrifuged at 3000 r/min at 5°C for 5 min. The organic phase was dried under a stream of nitrogen. The residue was dissolved in 0.2 mL of 10 mmol/L phosphate buffer (pH 6.1):acetonitrile (77:23), and the solution was injected into an HPLC system (Waters-2690) with a YMC-Pack Pro C18 column (4.6 i.d. × 150 mm, 5 μm), and a flow rate of 1 mL/min. UV absorbance was quantified at 230 nm. Detection limit was 5 ng/mL.

Statistical analysis

Intragastric pH is expressed as median values (ranges). Differences in between groups were assessed with the Wilcoxon signed-rank test. *P* values < 0.05 were considered to be statistical significant.

RESULTS

All 10 volunteers (all men; mean age, 28.7 years) completed the study according to the protocol. There were no adverse events. Three subjects were homo-EMs, 4 were hetero-EMs, and 3 were PM (Table 1).

Median intragastric pH values during the first 6 h after the administration of each drug are shown in Figure 1. The median intragastric pH was significantly higher with lafutidine 10 mg than with LPZ 30 mg 2 h, 3 h, 4 h, 5 h, and 6 h after drug administration.

Mean plasma drug concentrations during the first 6 h after treatment are shown in Figure 2. The time to peak plasma concentration (*T*_{max}) was shorter with lafutidine 10 mg (1 h) than with LPZ 30 mg (2 h).

DISCUSSION

PPIs and H₂RAs are potent agents widely used for the treatment of GERD. Recently, the frequency of GERD has been increasing in Japan. Endoscopic studies have shown that the overall prevalence of reflux esophagitis among Japanese adults is 14% to 16%^[3,4].

The increasing use of on-demand PPI therapy has raised various issues regarding efficacy. On-demand therapy has been reported an alternative to continuous treatment in patients with mild-to-moderate GERD who have frequent symptomatic relapses^[11,12]. Although many

clinicians regard PPIs to be superior to H₂RA in terms of continuous gastric acid suppression, a systematic review^[13] of the efficacy of PPIs for heartburn relief during the first 1 d to 2 d of therapy found that symptoms were completely relieved for the entire day in about 30% of patients after their first dose. In contrast, H₂RAs potently and quickly suppress gastric acid secretion^[17] and may thus have advantages over PPIs, especially for the on-demand treatment of GERD.

The incidence of atrophic gastritis in the general population is estimated to be higher in Japan than in western countries^[30,31], whereas gastric acid levels are generally lower in Japan^[18,19]. Moreover, endoscopic studies^[3,4] have reported that most Japanese patients have nonerosive reflux disease or mild forms of GERD. Consequently, some Japanese patients use H₂RAs rather than PPIs for the management of mild-to-moderate GERD. Against this background, we compared the H₂RA lafutidine with LPZ, one of the most widely used PPIs for the treatment of GERD in Japan.

In this study, lafutidine 10 mg was associated with a significantly prompter rise in intragastric pH and stronger inhibition of gastric acid secretion than was LPZ 30 mg during the early period (1-6 h) after administration of a single oral dose of either drug. Moreover, analysis of blood samples collected from randomly selected subjects showed that lafutidine 10 mg produced a significantly faster prompter rise in the plasma drug concentration than did LPZ 30 mg. These findings suggest that lafutidine 10 mg is especially useful for the on-demand treatment of acid-related symptoms in patients with mild GERD because of its prompter onset of action. However, we must consider the fact that H₂RA have a number of disadvantages as compared with PPI, including a shorter duration of action and the development of tachyphylaxis, limiting routine use^[10].

Our results are attributed to the different mechanisms of action of PPIs and H₂RAs. PPIs are absorbed in the small intestine and transported *via* the systemic circulation to gastric parietal cells, where they bind to the proton pump and potently inhibit gastric acid secretion^[32]. Some time is required for the PPIs to accumulate in parietal cells and then inhibit acid secretion. H₂RAs are absorbed in the small intestine, reach gastric cells *via* the systemic circulation, and then directly and rapidly bind to gastric cell histamine receptors, resulting in immediate inhibition of gastric acid secretion.

Inhibition of gastric acid secretion by PPIs is known to significantly depend on CYP2C19 genotype status, as well as on intrinsic pharmacokinetic and pharmacodynamic characteristics and dosing schemes^[20,21]. PPIs, such as LPZ, omeprazole, and pantoprazole, are mainly metabolized by CYP2C19 in the liver. As stated above, CYP2C19 genotypes are classified into the three groups: homo-EM, hetero-EM, and PM. Plasma PPI levels and intragastric pH values during PPI treatment are lowest in homo-EM, followed by hetero-EM, and highest in PM^[23-25]. Although the subjects this study included 3 PMs, lafutidine 10 mg was associated with a prompter rise in median intragastric pH during the early period (1-6 h) after administration of a single oral dose, as compared with LPZ 30 mg.

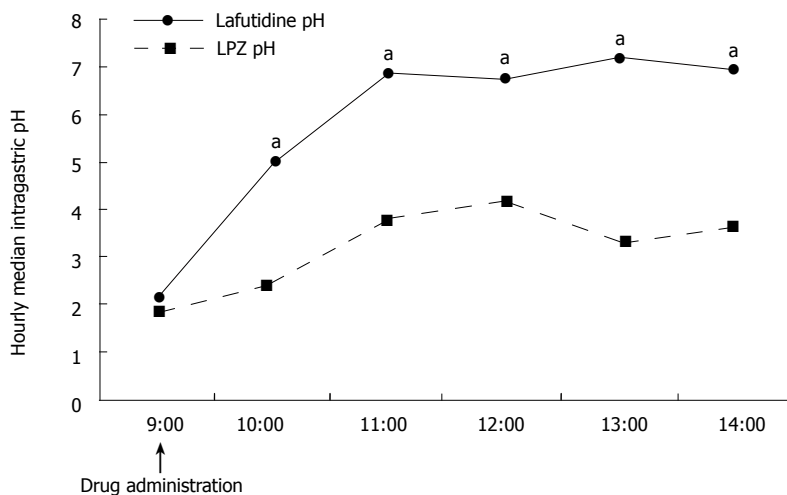


Figure 1 Median intragastric pH during a 6 h post administration period for all subjects ($n = 10$). The solid line (●) shows the hourly median intragastric pH after administration of 10 mg lafutidine, and the broken line (■) that after administration of 30 mg LPZ. The median intragastric pH values during the post administration period were significantly higher with lafutidine than with LPZ 2, 3, 4, 5, and 6 h after drug administration. Arrow: drug administration. The statistical significance of differences between the drug groups in intragastric pH among was assessed with the Wilcoxon signed-rank test. $^aP < 0.05$ vs LPZ pH.

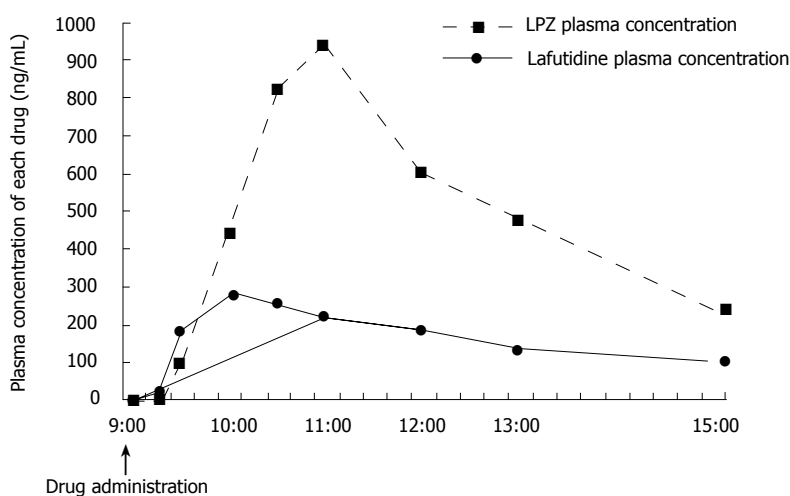


Figure 2 Plasma drug concentrations during a 6 h post administration period in four individuals (No. 1, 4, 5, 6). The solid line (●) indicates the plasma drug concentration after the administration of 10 mg lafutidine, and the broken line (■) that after the administration of 30 mg LPZ. Lafutidine (T_{max} 1 h) reached its peak plasma concentration faster than LPZ (T_{max} 2 h). Arrow: Drug administration.

In conclusion, lafutidine 10 mg has a prompt onset of action than LPZ 30 mg in the early phase (1–6 h) after administration of a single oral dose. Lafutidine may thus offer advantages over LPZ for the on-demand treatment of GERD.

COMMENTS

Background

The prevalence of Gastroesophageal reflux disease (GERD) symptoms is now increasing in Japan. Concerning the characteristics of GERD in Japan, it is important to distinguish the significant difference of acid secretion in Japanese patients when compared with that of patients from western countries. Acid secretion among the Japanese individuals lower when compared to that of the western population irrespectively of *H. pylori* infection status. In Japan, histamine H_2 receptor antagonists (H_2 RAs) are thus sometimes used for the treatment of mild-to-moderate GERD.

Research frontiers

To compare lafutidine 10 mg (H_2 RAs) to LPZ 30 mg (proton pump inhibitors: PPI) in the antisecretory activity and blood drug concentration in the immediate early period after administration of a single dose.

Innovations and breakthroughs

Few studies have reported the effect at the early post-administration phase (1–6 h) of single dose of H_2 RA and PPI.

Applications

We clearly state that lafutidine 10 mg has a prompt onset of action than LPZ

30 mg in the early phase (1–6 h) after administration of a single oral dose. It is reported that rapid acid suppression is important for effective pain relief at the onset of treatment in GERD patients. Thereby, our results show that lafutidine offers advantages over LPZ for the on-demand treatment of GERD.

Peer review

This is a nice clear study. The authors ascertained the effectiveness of lafutidine compared with LPZ in the elevation of intragastric pH in the immediate early period after a single oral administration.

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Association between colonic polyps and diverticular disease

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Abstract

AIM: To evaluate the association between colonic polyps and diverticular disease in Japan.

METHODS: We retrospectively reviewed the medical records of 672 consecutive patients who underwent total colonoscopy between August 2006 and April 2007 at Nishinjo Hospital, Okinawa, Japan. Patients with a history of any of the following were excluded from the study: previous polypectomy, colonic resection, and inflammatory bowel diseases. The association between colonic polyps and diverticular disease was analyzed by logistic regression analysis, adjusted for age and sex.

RESULTS: Prevalence of colonic polyps in all patients with diverticular disease was significantly higher than that in those without diverticular disease (adjusted odds ratio 1.7).

CONCLUSION: Our data showed that patients with diverticular disease have a higher risk of colonic polyps compared to those without.

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Key words: Colonic polyps; Colonic neoplasm; Diverticular disease; Proximal diverticular disease; Colonoscopy

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INTRODUCTION

The prevalence of colonic neoplasia and diverticular disease has increased in recent years^[1-5]. Both have common risk factors such as age and a lack of dietary fiber^[6-10]. Despite common epidemiological trends and risk factors, any association between these diseases has not been clarified. Although some data have been reported regarding the association between these diseases in Western countries^[11-14], there has been no study in Japan. There are differences between diverticular disease in Western countries and that seen in Asia, including Japan^[15-19]. Diverticular disease of the right colon is rare in Western countries^[20-22], whereas in Asia, diverticular disease of the right colon is common and has been increasing in recent years^[23-27].

This study evaluated the association between colonic polyps and diverticular disease in Japanese patients undergoing total colonoscopy.

MATERIALS AND METHODS

We retrospectively reviewed the medical records of consecutive patients who underwent total colonoscopy between August 2006 and April 2007 at Nishinjo Hospital, Okinawa, Japan. The major indications for total colonoscopy were screening examination after hemorrhoidectomy and rectal bleeding. Patients with a history of any of the following were excluded from this study: Previous polypectomy, colonic resection, and inflammatory bowel diseases. The location of diverticula and polyps was classified into three groups: Distal, proximal and bilateral colon. The borderline between the distal and proximal colon was set at the splenic flexure. Diverticular disease was defined as the presence of one

Table 1 Number of patients with diverticular disease and colonic polyps by colon segment *n* (%)

Colon segment	Patients with diverticular disease (%)	Patients with polyps (%)
Distal colon	20 (3.0)	89 (13.2)
Proximal colon	98 (14.6)	53 (7.9)
Bilateral colon	47 (7.0)	47 (7.0)
Total	165 (24.5)	189 (28.1)

Table 2 Comparison of demographic features between patients with or without diverticular disease

	Patients with diverticular disease	Patients without diverticular disease
No. of patients	165	508
Mean age (SD)	58.0 (13.6) ^b	47.3 (14.7)
Sex (female: male)	52:113 ^d	219:289
No. of patients with polyps (%)	71 (43.0) ^d	118 (23.2)

^b*P* < 0.01 *vs* without diverticular disease using the *t* test; ^d*P* < 0.01 *vs* without diverticular disease using the chi-square analysis.

or more diverticula, and all polyps were diagnosed as adenoma by histological examination.

Statistical analysis

The chi-square test was used to compare sex and prevalence of colonic polyps, and the *t* test to compare mean age. Logistic regression analysis was used to examine the association between diverticular disease and colonic polyps, adjusting for age and sex. *P* < 0.05 was considered statistically significant. All statistical analyses were performed with SPSS 15.0 for Windows.

RESULTS

The present study included 672 consecutive patients. Of these, 165 (24.5%) had diverticular disease and 189 (28.1%) had colonic polyps. The most common segment for diverticular disease was the proximal, followed by the bilateral and distal colon. The most common segment for colonic polyps was the distal, followed by the proximal and bilateral colon (Table 1). Among patients with diverticular disease, none had active segmental colitis.

Table 2 summarizes the demographic features of patients with or without diverticular disease. The mean age of patients with diverticular disease was significantly higher than that of patients without diverticular disease (*P* < 0.001). There were significantly more males in those patients with diverticular disease than those without (*P* = 0.008). The prevalence of colonic polyps in patients with or without diverticular disease was significantly different at 43% and 23.2%, respectively.

Using logistic regression analysis adjusted for age and sex, we calculated the adjusted odds ratio (OR) for colonic polyps (Table 3). This confirmed that the prevalence of colonic polyps in all patients with diverticular disease or those with diverticular disease in the proximal colon was significantly higher than that in

Table 3 Association between colonic polyps and diverticular disease adjusted for age and sex by logistic regression analysis

Segment with diverticular disease	No. of patients with polyps /No. of patients with diverticular disease (%)	OR	95% CI	<i>P</i> values
Distal colon	11/20 (55.0)	2.3	0.9-5.8	0.09
Proximal colon	41/98 (41.8)	1.9	1.2-3.0	0.01
Bilateral colon	19/47 (40.4)	1.2	0.6-2.3	0.60
Total	71/165 (43.0)	1.7	1.1-2.5	0.01

patients without diverticular disease (adjusted OR 1.7 and 1.9, respectively).

DISCUSSION

Colonic neoplasia and diverticular disease have common epidemiological trends and risk factors such as age and a lack of dietary fiber^[6,7]. However, little is known about any association between these diseases. Morini and others found an increased risk for sigmoid colon adenoma in Italian patients with diverticular disease, in a prospective study^[28]. Kieff and others have reported an increased risk for distal neoplasia in women in the USA with extensive distal diverticulosis, in a cross-sectional study^[29]. Although the sample size and distribution of patients included in the present study might inadequately reflect the general population of Japan, our data showed a 1.7-fold increased risk for colonic polyps in patients with diverticular disease, as compared to those without. In addition, although the prevalence of colonic polyps in patients with diverticular disease in the proximal colon and that in patients without was significantly different, the prevalence of colonic polyps in patients with diverticular disease in the distal or bilateral colon and that in patients without diverticular disease was not significantly different. This observation may be the result of the limited number of patients with diverticular disease in the distal and bilateral colon. However, this result was similar to a previous study in Korea, in which patients with proximal diverticular disease had a higher risk of any proximal neoplasia than did other patients^[30]. Diverticular disease of the proximal colon is rare in Western countries, whereas in Asia including Japan, diverticular disease of the proximal colon is relatively common^[16,17,23,24]. These results suggest that, regardless of the segment with diverticular disease or race, patients with diverticular disease have a higher risk of colonic neoplasia.

In conclusion, our data showed patients with diverticular disease have a higher risk of colonic polyps compared to those without (OR 1.7). This finding needs to be taken into account in surveillance for colonic neoplasia. However, further research is needed to clarify the mechanism of the association between these diseases.

COMMENTS

Background

Prevalences of colonic neoplasia and diverticular disease have increased in recent years. Both colonic neoplasia and diverticular disease have common risk factors such as age and a lack of dietary fiber. Despite common epidemiological trends and risk factors, any association between these diseases has not been clarified.

Research frontiers

There is an increasing body of epidemiological evidence regarding an association between diverticular disease and colonic polyps.

Innovations and breakthroughs

This study clarified the strong association between diverticular disease and colonic polyps. Moreover, this study suggested that regardless of the segment with diverticular disease or race, patients with diverticular disease have a higher risk of colonic neoplasia.

Applications

These results need to be taken into account in surveillance for colonic neoplasia.

Peer review

It is interesting that in the authors' series there were similar associations between left and right sided diverticulosis and polyps.

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RAPID COMMUNICATION

Comparison of immediate surgical outcomes between posterior pelvic exenteration and standard resection for primary rectal cancer: A matched case-control study

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Abstract

AIM: To determine the immediate surgical outcome and recovery of bowel function following posterior pelvic exenteration (PPE) for primary rectal cancer with suspected local invasion to the female internal reproductive organs, in comparison with a case-control series of standard resection for primary rectal cancer.

METHODS: We analyzed 10 consecutive female patients undergoing PPE for the aforementioned indication between December 2003 and May 2006 in a single institution. Data were prospectively collected during hospitalization, including patient demographics, tumor- and operation-related variables and early surgical outcomes. These patients were compared with a group of female patients, matched for age, co-morbidity and location of tumor, who underwent standard resection for primary rectal cancer in the same period (non PPE group).

RESULTS: In the PPE group, pathological reports showed direct invasion of the reproductive organs in 4 cases and an involvement of lymph nodes in 7 cases. A sphincter-saving operation was performed in each case. Operative time was longer (274 min *vs* 157 min, $P < 0.001$) and blood loss was greater (769 mL *vs* 203 mL, $P = 0.008$) in the PPE group. Time to first bowel movement, time to first defecation, time to resumption of normal diet, and hospital stay were not significantly different between the two groups. Postoperative complication rates were also similar.

CONCLUSION: PPE for rectal cancer was associated

with longer operative time and increased blood loss, but did not compromise immediate surgical outcomes and postoperative bowel function compared to standard rectal resection.

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Key words: Pelvic exenteration; Rectal cancer; Outcomes; Morbidity; Postoperative bowel function

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INTRODUCTION

The incidence of rectal cancer invading the female reproductive organs has been reported to be up to 18%^[1-3]. An en-bloc resection of the rectum, uterus and both ovaries, known as a posterior pelvic exenteration (PPE), is a curative procedure for this condition^[4-6]. There is also certainty that PPE should be performed in every female rectal cancer patient with suspected local invasion to the reproductive organs because it is difficult to distinguish intraoperatively whether adherence to the adjacent organs is malignant or only due to the peritumoral inflammatory process^[2,7]. Non en-bloc resection of the tumor invading other structures resulted in higher risk of local recurrence and poorer survival^[7].

PPE remains a radical procedure associated with significant morbidity and mortality^[8-10]. Details of early surgical outcomes related to the procedure are not clearly defined in the available literature. Furthermore, most investigations^[11-16] included heterogeneous patients with different surgical operations, including cases of primary and recurrent rectal cancer, and focused primarily only on patient survival rate.

The aim of this study was to determine the immediate surgical outcome and recovery of bowel function following PPE for primary rectal cancer with suspected local invasion to the female internal reproductive organs in comparison with a case-control series of standard resection for primary rectal cancer.

MATERIALS AND METHODS

Patients

We carried out an analysis of 10 consecutive female patients with rectal adenocarcinoma with suspected local invasion to the female internal reproductive organs who underwent PPE between December 2003 and May 2006 at the Department of Surgery, Faculty of Medicine Siriraj Hospital, Bangkok, Thailand. Data were prospectively collected during hospitalization. The study was approved by the Institutional Ethics Committee and informed consent was obtained from all the patients. These patients were compared with a group of female patients who underwent standard resection for primary rectal cancer in the same period (non PPE group). They were matched for age, co-morbidity and location of tumor, with the ratio of 1 PPE case to 2 non PPE cases.

Rectal cancer was defined as a tumor with the lower edge located within 15 cm from the anal verge measured by rigid sigmoidoscopy. PPE was defined as an extirpation of the rectum in block with the internal genital organs and draining lymph nodes. Patients receiving neoadjuvant therapy, or who had recurrent tumor or laparoscopic resection, were excluded.

Surgical procedure

All patients were operated on by the same surgical team in the colorectal unit. Gynecologists participated in a few cases with PPE. Each patient underwent preoperative mechanical bowel preparation using 2 liters of polyethylene glycol a day before surgery. In the operating room, all patients received general anesthesia with or without epidural anesthesia. Intravenous prophylactic antibiotics were administered. The abdominal operation was performed *via* midline incision. Standard oncological resection was performed in every patient. Adequate mesorectal excision was performed in tumors of the upper third of the rectum and total mesorectal excision in tumors of the middle and lower third of the rectum. The type of reconstruction, either straight, side-to-end or colorectal anastomosis, was left to the discretion of each surgeon. No protective stoma and pelvic drain was performed. Prophylactic intravenous antibiotics were discontinued within 24 h-48 h.

Statistical analysis

All data were recorded including patient demographic, operative details (suspected organs involved, operative time and blood loss), pathological staging and postoperative outcomes. The postoperative outcomes, including mortality, morbidity, time to first bowel movement, time to first defecation, time to resumption of normal diet, and hospital stay, were analyzed. Patients were discharged from

Table 1 Correlation between indications, tumor location and pathologic findings in PPE group (*n* = 10)

Suspected organs involved	<i>n</i> (lower: middle: upper rectum)	Pathological findings		
		Direct invasion (T4)	Perirectal invasion (T3)	Positive lymph nodes (N-positive)
Vagina	1 (1:0:0)	0	1	1
Uterus	7 (0:5:2)	2	5	4
Ovaries	2 (0:1:1)	2 ¹	0	2
Total	10 (1:6:3)	4	6	7

¹Including 1 case of Krukenberg tumor.

the hospital when they had no fever, good ambulation, good appetite and satisfactory recovery of bowel function. All patients were scheduled for follow-up at 30 d postoperatively. Patients with T3/4 or N-positive tumors were further scheduled for adjuvant therapy.

All data were prepared and compiled using SPSS computer software (version 10.0 for Windows). Means and standard deviations were assessed. The Kolmogorov-Smirnov test was used to test for the pattern of data distribution. *t*-tests were used to compare data between the two groups when they showed normal distribution. Mann-Whitney *U* tests were used when data were not normally distributed. Pearson chi-square tests or Fisher's exact tests were used for categorical data. A *P* value of less than 0.05 was considered statistically significant.

RESULTS

During the 2.5-year period mentioned, 64 female patients underwent various curative resection of the rectum for primary rectal cancer. Ten patients (16%), with a mean age of 57 years (range 43-81), underwent PPE. Sphincter-saving operations and R0 resection were achieved in all cases studied.

In the PPE group, pathological reports showed direct invasion of reproductive organs in 4 cases and an involvement of lymph nodes in 7 cases. Correlation between indications, tumor location and pathologic findings in the PPE group are shown in Table 1. Patient- and tumor- related variables were not statistically different between the PPE and non-PPE groups. The average operative time and blood loss were significantly greater in the PPE group (274 min *vs* 157 min, *P* < 0.001; 769 mL *vs* 203 mL, *P* = 0.008, respectively; Table 2).

No thirty-day postoperative mortality occurred in this study. Each group had a complication rate of 10%; one intraabdominal abscess requiring a 10-d regimen of intravenous antibiotics occurred in the PPE group, one superficial surgical site infection and one anastomotic leakage requiring percutaneous drainage and bowel rest occurred in the non-PPE group. Time to first bowel movement, time to first defecation, time to resumption of normal diet and postoperative hospitalization were not significantly different between the two groups (Table 3).

DISCUSSION

In the present series, PPE was employed in 16% of the

Table 2 Patient- and tumor- related variables between PPE group and non PPE group (mean \pm SD)

Variables	PPE (n = 10)	Non PPE (n = 20)	P
Patient related			
Age (yr)	57.4 \pm 13.5	57.8 \pm 13.1	0.94
BMI (kg/m ²)	22.2 \pm 2.9	21.4 \pm 3.7	0.58
ASA status I : II : III	30:50:20	45:50:05	0.39
Hypoalbuminemia ¹	50	25	0.23
Preoperative CEA (ng/mL)	36.9 \pm 44.7	11.5 \pm 21.0	0.12
Tumor related			
Stage I : II : III : IV ²	0:30:60:10	20:20:60:0	0.30
Tumor size (cm)	6.0 \pm 2.2	4.8 \pm 2.0	0.16
Location of the tumor from the anal verge (cm)	9.1 \pm 3.3	9.0 \pm 3.0	0.93

BMI: Body mass index; ASA: American society of anesthesiologists; CEA: Carcinoembryonic antigen. ¹Serum albumin less than 3.5 mg/dL; ²Patient with Krukenberg tumor.

female patients with primary rectal cancer, which was comparable to previous reports of 14%-28% in the literature^[1,7,17]. The mortality rate after PPE is generally less than 10%^[1,13,18,19], depending on patient characteristics, comorbidity and hospital setting. The zero 30-d mortality found in the present study may be the result of the fact that all the operations were electively performed by experienced colorectal surgeons and few patients had severe co-morbidity. In order to minimize mortality, PPE should be performed in selective cases in a specialized and well-equipped medical institution.

The PPE group tended to have larger tumors and a higher preoperative carcinoembryonic antigen (CEA) level than the non PPE group. However, this finding did not decrease the rate of sphincter-saving operation because preservation of the anal sphincter mainly depends on the location of the tumor in relation to the anal verge rather than the size of the lesion^[20,21]. In our experience, PPE increases the operative time and blood loss but does not increase morbidity and hospital stay time. Bannura *et al*^[1] revealed that PPE was associated with prolonged operative time, increased postoperative complications and delayed hospital discharge when compared with non PPE cases. This report from Chile included 50% morbidity with an average hospital stay of 19 d in the PPE group. The high morbidity in that study may be due to one-third of PPE involving abdominoperineal excision and one-fifth receiving neoadjuvant chemoradiation. Operation for recurrent pelvic malignancy^[13], concomitant resection of bony pelvis^[16], and preoperative pelvic radiation^[22] have been identified as risk factors for postoperative morbidity after PPE. The common complications following PPE include intraabdominal hemorrhage, pelvic abscess or fluid collection, anastomotic leakage, urinary tract infection, wound infection, and intraperineal fistula^[23,24].

In the present study, we found no difference in clinical recovery of bowel function between the two groups. There have been a number of independent factors influencing recovery of gut function^[25-27], including postoperative complications, narcotics administration, electrolyte imbalance, and hypoalbuminemia^[28]. However,

Table 3 Operation related variables and surgical outcomes between PPE group and non PPE group (mean \pm SD)

Variables	PPE (n = 10)	Non PPE (n = 20)	P value
Operation related			
Operative time (min)	274 \pm 73	157 \pm 62 ^b	< 0.001
Blood loss (mL)	769 \pm 549	203 \pm 136 ^b	0.008
Outcomes			
Complications	10	10	1.00
Time to first bowel movement (h)	70 \pm 29	59 \pm 26	0.31
Time to first defecation (d)	5.0 \pm 1.2	4.6 \pm 1.5	0.42
Time to resumption of normal diet (d)	5.8 \pm 3.0	4.6 \pm 0.9	0.09
Hospital stay (d)	11.5 \pm 7.2	9.2 \pm 4.1	0.26

^bP < 0.01.

no published study has been able to demonstrate any correlation between prolonged operative time or increased blood loss and delayed recovery of postoperative bowel function^[29]. Length of hospital stay for the PPE group and non-PPE group was not significantly different. This finding may suggest that additional non-gastrointestinal resection does not increase operative risk and hospital stay in rectal cancer surgery^[30].

In conclusion, PPE was associated with longer operative time and increased blood loss, but did not compromise immediate surgical outcomes and postoperative bowel function compared to standard rectal resection. PPE can be justified as a liberal and safe operation for primary rectal cancer with suspected local invasion to the female internal reproductive organs.

COMMENTS

Background

Posterior pelvic exenteration (PPE) is the gold standard operation for rectal cancer invading the female reproductive organs. There is also no doubt that PPE should be performed in every female rectal cancer patient with suspected local invasion to the reproductive organs because it is difficult to distinguish intraoperatively whether adherence to the adjacent organs is malignant or only peritumoral inflammatory process. However, PPE remains a radical procedure and could be associated with a significant morbidity and mortality.

Research frontiers

Details of early surgical outcomes following PPE are not clearly defined in available literatures. Furthermore, most investigations included heterogeneous patients with different surgical operations, and both with cases of primary and of recurrent rectal cancer.

Innovations and breakthroughs

The present study clearly demonstrated that PPE did not compromise immediate surgical outcomes and postoperative bowel function comparing with standard rectal resection for rectal cancer.

Applications

PPE could be justified as a liberal and safe operation for primary rectal cancer with suspected local invasion to the female internal reproductive organs although it is associated with longer operative time and increased blood loss comparing with standard rectal resection. Further research might focus on the long-term outcomes (such as disease free survival and survival time) between the two procedures in female patients with locally advanced rectal cancer.

Peer review

This clinical study compares the short-term surgical outcomes between posterior pelvic exenteration and standard rectal resection. The manuscript is well-written and very informative. It has an impact on surgical management for locally advanced rectal cancer.

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RAPID COMMUNICATION

Alcohol consumption and metabolic syndrome among Shanghai adults: A randomized multistage stratified cluster sampling investigation

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Abstract

AIM: To examine the relations of alcohol consumption to the prevalence of metabolic syndrome in Shanghai adults.

METHODS: We performed a cross-sectional analysis of data from the randomized multistage stratified cluster sampling of Shanghai adults, who were evaluated for alcohol consumption and each component of metabolic syndrome, using the adapted U.S. National Cholesterol Education Program criteria. Current alcohol consumption was defined as more than once of alcohol drinking per month.

RESULTS: The study population consisted of 3953 participants (1524 men) with a mean age of 54.3 ± 12.1 years. Among them, 448 subjects (11.3%) were current alcohol drinkers, including 405 males and 43 females. After adjustment for age and sex, the prevalence of current alcohol drinking and metabolic syndrome in the general population of Shanghai was 13.0% and 15.3%, respectively. Compared with non drinkers, the prevalence of hypertriglyceridemia and hypertension was higher while the prevalence of abdominal obesity, low serum high-density-lipoprotein cholesterol (HDL-C) and diabetes mellitus was lower in subjects who consumed alcohol twice or more per month, with a trend toward reducing the prevalence of metabolic syndrome. Among the current alcohol drinkers, systolic blood pressure, HDL-C, fasting

plasma glucose, and prevalence of hypertriglyceridemia tended to increase with increased alcohol consumption. However, low-density-lipoprotein cholesterol concentration, prevalence of abdominal obesity, low serum HDL-C and metabolic syndrome showed the tendency to decrease. Moreover, these statistically significant differences were independent of gender and age.

CONCLUSION: Current alcohol consumption is associated with a lower prevalence of metabolic syndrome irrespective of alcohol intake (g/d), and has a favorable influence on HDL-C, waist circumference, and possible diabetes mellitus. However, alcohol intake increases the likelihood of hypertension, hypertriglyceridemia and hyperglycemia. The clinical significance of these findings needs further investigation.

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Key words: Alcohol; Metabolic syndrome; Obesity; Type 2 diabetes; Epidemiology; Chinese

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INTRODUCTION

Alcohol consumption is a double-edged sword, and perhaps no other factor in cardiovascular health is capable of cutting so deeply in either direction depending on how it is used. Accumulating scientific evidence indicates that light to moderate alcohol consumption is associated with a lower cardiovascular mortality and a reduced risk of developing type 2 diabetes mellitus^[1-6]. Some of the biological mechanisms reported to explain this observation include an improvement in lipid profile, especially high-density-lipoprotein-cholesterol (HDL-C) and increased insulin sensitivity^[1-6]. In contrast, heavy or risky drinking is toxic to both the heart and the overall health and is the third

leading cause of premature death among Americans^[1].

Metabolic syndrome is a clustering of low serum HDL-C, elevated serum triglycerides, hyperglycemia, abdominal obesity, and elevated blood pressure, mediated in part by insulin resistance. Metabolic syndrome is associated with an increased risk of developing diabetes mellitus and cardiovascular disease^[7]. Alcohol consumption has a favorable influence on selective components of metabolic syndrome, contributing to the reduction in risk of developing metabolic syndrome in the U.S. population^[8-10]. However, the validity of its putative benefits to metabolic disorders has not been well evaluated in Chinese.

The present study was to investigate the prevalence of current alcohol drinking and metabolic syndrome among Shanghai adults, and to explore the relationship between alcohol consumption and components of metabolic syndrome.

MATERIALS AND METHODS

Survey design and study sample

We assigned a number to each of the 16 urban districts of Shanghai and selected two districts at random (Yangpu District and Pudong New District). Of the 11 residential districts within Yangpu and Pudong, we randomly selected the Pingliang and Shanggang residential districts, containing 30 and 26 neighborhood communities, respectively. From these, we selected eight neighborhood communities in total. Resident groups were randomly selected from each sample neighborhood community. From October 2002 to April 2003, investigations were conducted in adults aged 20 years or more in the selected resident groups at home. We excluded individuals from the study if they had a history of malignancy and other severe diseases.

This program was approved by the Research Ethics Committee of the Shanghai Health Bureau and all participants provided their written informed consent prior to their inclusion in the study. General physical examinations and laboratory assessments were performed for each study subject at a mobile examination center following an overnight fast of at least 12 h.

Data collection

Interview: Selected individuals were interviewed at their homes using a self-designed questionnaire that gathered information on demographic characteristics, medical history, medications and health-related habits. Consumption of alcohol was ascertained from a series of questions including whether the respondent consumed 12 drinks (one drink is considered to contain 10 g alcohol) in the past 12 mo. If so, respondents were asked to quantify the number of days they consumed alcohol over the past 1 year and the number of drinks per day on drinking days. From these data, we calculated an average daily intake of alcohol. The questionnaire was pre-tested in the population prior to the study.

Physical examination: Body weight of the participants was measured in light clothing and without shoes to the nearest half kilogram. Their height was measured to the nearest half centimeter. Body mass index (BMI) was

calculated as weight (kg) divided by height squared (m^2). Waist circumference (to the nearest half centimeter) was measured at the mid-point between the lower border of the rib cage and the iliac crest, whereas hip circumference was similarly obtained at the widest point between the hip and buttock, enabling calculation of the waist-to-hip ratio. Three blood pressure readings were obtained at intervals of one min. The second and third systolic and diastolic pressure readings were averaged and used in the analyses.

Laboratory assessments: Venous blood samples were collected at 0 and 120 min following a 75 g oral glucose challenge for non-diabetics or 100 g steamed bread for diabetics. Samples were centrifuged at 2000 g for 10 min at 25°C immediately, frozen and shipped to a central laboratory of the Shanghai Center for Disease Control and Prevention, where they were stored initially at -20°C and then at -70°C. Subsequently, serum glucose was determined using a modified hexokinase method. Fasting serum total cholesterol (TC) and triglyceride concentrations were measured enzymatically by color absorptiometry based on a peroxidase-catalysed reaction. HDL-C was measured after precipitation of other lipoproteins with a polyanion/divalent cation mixture. Low-density-lipoprotein-cholesterol (LDL-C) was calculated from the measured values of TC, triglycerides and HDL-C using the following formula: $LDL = (TC) - (HDL) - (triglycerides/5)$. LDL was not calculated if the triglyceride concentration was > 4.52 mmol/L. All these serum biochemistries were performed using a Bayer model 1650 automated bio-analyzer (Bayer Diagnostic, Basingstoke, UK).

Quality control: Field researchers were recruited from Shanghai Center for Disease Control and Prevention, and Shanghai Jiaotong University School of Medicine. Before the investigation, the researchers were given systematic training to ensure standardization of the investigation procedure. For further quality control, 5% of questionnaires and blood samples were re-examined. Kappa analysis of these samples showed a good consistency in the diagnostic test (data not shown).

Definitions: Obesity and abdominal obesity were categorized according to the new BMI criteria for Asians by the regional office for Western Pacific Region of the World Health Organization (WHO)^[11]. Hypertension was defined as given in the Seventh Report of the Joint National Committee on Prevention, Detection, Evaluation, and Treatment of High Blood Pressure (JNC7)^[12]. Diagnoses of impaired fasting glucose (IFG), impaired glucose tolerance (IGT) and type 2 diabetes were based on WHO criteria published in 1999 (WHO/NCD/NCS/99.2)^[13,14]. Dyslipidemia (including hypertriglyceridemia and low HDL-C) and metabolic syndrome were diagnosed on the basis of the adapted U.S. National Cholesterol Education Program's Adult Treatment Panel III (NCEP ATP III) criteria with the exception of abdominal obesity (waist circumference 90 cm in men and 80 cm in women)^[7,15]. We regarded participants who reported current use of antihypertensive or anti-diabetic medications or fibrates as participants with a high blood pressure or diabetes or hypertriglyceridemia, respectively.

Current alcohol consumption was defined as more than once of alcohol drink per month. The participants were then divided into current alcohol drinkers and current non drinkers, the former were further classified into light drinkers (1-9.9 g/d), moderate drinkers (10-29.9 g/d) and excessive drinkers (≥ 30 g/d) according to their average daily alcohol intake in the past 12 mo^[1,9,10,16].

Statistical analysis

All data were analyzed using SPSS 11.0 software (SPSS, Chicago, IL, USA). Unpaired *t*-test, χ^2 contingency test, Fisher's exact test and trend analysis were performed whenever appropriate. Non-parametric methods were also used for abnormally distributed values. Some analyses were adjusted for age and sex. Kappa analysis was performed for blood biochemical data as a quality control. All *P* values provided are for two-sided tests. *P* < 0.05 was considered statistically significant.

RESULTS

Sampling status and general data

We used a stratified, multistage probability cluster sampling method to obtain a representative sample from the Shanghai non-institutionalized population. The neighborhoods investigated contained 3953 residents aged 20 years or more as subjects in the present study, corresponding to approximately 2.99/10 000 of the Shanghai population according to the data collected in the Fifth China National Census in 2002 (<http://www.china-un.ch/eng/ljzg/shjjtj/t85845.htm>).

The study population included 1524 males and 2379 females (excluding pregnant women), giving a male to female ratio of 1:1.56. Their age ranged 20-88 years and no significant difference was noted between the ages of male and female subjects. In comparison with the sex and age obtained from the Fifth National Census of Shanghai, the study population contained a higher percentage of elderly subjects and women (*P* < 0.01). Therefore, some of the results were adjusted for age and sex in order to better represent the real situation in Shanghai.

Prevalence of current drinking and metabolic syndrome

Of the 3953 enrolled subjects, 448 (11.3%) had a history of current alcohol consumption, men accounted for 26.6% and women 1.8% (*P* < 0.001). As shown in Table 1, the prevalence of current alcohol drinking both in overall subjects and in males increased with age and reached its peak at the age of 40-49 years. However, no significant changes were found in females. After adjusted for age and sex, the prevalence of current alcoholic drinking among Shanghai adults was found to be 13.0%, which was significantly higher in males than in females (24.5% *vs* 1.5%, *P* < 0.001).

Among the 448 participants with a history of current alcohol drinking, over two-thirds (72.1%) of them were light to moderate drinkers, light to moderate female drinkers accounted for 95%. There was no difference in age among different drinking groups (Table 2). Since only 125 (27.9%) subjects were found to be excessive drinkers in the

Table 1 Sex and age related prevalence of current alcohol drinking in 3953 adults in Shanghai *n* (%)

Age (yr)	Total	Current drinking	Male	Current drinking	Female	Current drinking
20-29	139	8 (5.8)	61	7 (11.5)	78	1 (1.3)
30-39	157	16 (10.2)	65	15 (23.1)	92	1 (1.1)
40-49	895	118 (13.2)	306	108 (35.3)	589	10 (1.7)
50-59	1305	167 (12.8)	477	153 (32.1)	828	14 (1.7)
60-69	794	77 (9.7)	327	67 (19.1)	467	10 (2.1)
≥ 70	663	62 (9.4)	288	55 (19.1)	375	7 (1.9)
Total	3953	448 (11.3)	1524	405 (26.6)	2379	43 (1.8)

Current alcohol drinking is defined as more than once of alcohol drinking per mo in the past one year.

Table 2 Classification of 448 subjects with current alcohol drinking according to alcohol consumption *n* (%)

	Light drinking	Moderate drinking	Excessive drinking
Total (<i>n</i> = 448)	180 (40.2)	143 (31.9)	125 (27.9)
Male (<i>n</i> = 405)	149 (36.8)	133 (32.8)	123 (30.4)
Female (<i>n</i> = 43)	31 (72.1)	10 (23.3)	2 (4.7)
Age (yr)	54.47 \pm 10.80	55.35 \pm 11.62	55.05 \pm 9.84
Alcohol intake (g/d)	2.60 \pm 2.84	17.95 \pm 5.07	58.30 \pm 80.13

Light drinking is defined as average alcohol consumption of less than 10 g/d, moderate drinking as average daily alcohol consumption of 10-30 g, and excessive drinking as average alcohol consumption of equal to or more than 30 g/d in the past 12 mo.

study, their average daily alcohol consumption was 58 g, and few of them were heavy or risky drinkers (alcohol consumption > 40 g/d), so we did not further distinguish heavy drinkers from excessive drinkers.

The prevalence of obesity, abdominal obesity, hypertension, hypertriglyceridemia, low serum HDL-C, diabetes mellitus and metabolic syndrome in the 3953 study subjects was 43.3%, 35.4%, 49.3%, 25.7%, 21.3%, 14.8% and 23.9%, respectively. After adjusted for age and sex, the prevalence of metabolic syndrome among adults in Shanghai was 15.3% according to the adapted NCEP-ATP III criteria^[17].

Effects of current alcohol consumption on metabolic syndrome

The effects of current alcohol drinking on metabolic syndrome features in 3953 Shanghai adults are shown in Table 3. The blood pressure, serum concentration of triglyceride, HDL-C and FPG, prevalence of hypertension and hypertriglyceridemia were higher while the prevalence of abdominal obesity, low serum HDL-C and diabetes mellitus were lower in alcoholic drinkers than in non-alcoholic drinkers. However, the prevalence of metabolic syndrome in alcoholic drinkers only decreased mildly compared with the non-alcoholic drinkers. When adjusted for sex and age, the differences in metabolic syndrome features still existed between the two groups.

Among the 448 subjects with current alcohol consumption, the trend analysis showed that systolic blood pressure (SBP), serum HDL-C and FPG, and prevalence of hypertriglyceridemia increased progressively, while se-

Table 3 Effects of current alcohol drinking on metabolic syndrome features in 3953 Shanghai adults

	Non-drinking (<i>n</i> = 3505)	Current drinking (<i>n</i> = 448)	<i>P</i> value
Age (yr)	54.2 ± 12.4	54.9 ± 10.8	> 0.05
BMI (kg/m ²)	24.6 ± 4.39	24.8 ± 3.84	> 0.05
Waist-to-hip ratio	0.84 ± 0.14	0.85 ± 0.15	0.12
SBP (mmHg)	129.0 ± 18.8	132.8 ± 17.2	< 0.001
DBP (mmHg)	81.8 ± 11.9	85.8 ± 10.4	< 0.001
Triglyceride (mmol/L)	1.36 ± 1.04	1.62 ± 1.38	< 0.001
Total cholesterol (mmol/L)	4.98 ± 0.97	4.91 ± 0.93	0.15
HDL-C (mmol/L)	1.49 ± 0.40	1.57 ± 0.41	< 0.001
LDL-C (mmol/L)	2.90 ± 0.82	2.73 ± 0.83	< 0.001
FPG (mmol/L)	5.77 ± 1.60	5.98 ± 1.70	< 0.01
Obesity, <i>n</i> (%)	1512 (43.1)	199 (44.2)	0.67
Abdominal obesity, <i>n</i> (%)	1270 (36.2)	129 (28.8)	< 0.01
Hypertension, <i>n</i> (%)	1525 (43.5)	246 (45.9)	< 0.001
Hypertriglyceridemia, <i>n</i> (%)	874 (24.9)	140 (31.3)	< 0.001
Low HDL-C, <i>n</i> (%)	770 (22.0)	72 (16.1)	< 0.05
IFG/IGT, <i>n</i> (%)	950 (27.1)	118 (26.3)	0.24
Diabetes mellitus, <i>n</i> (%)	540 (15.4)	45 (10.0)	< 0.01
Metabolic syndrome, <i>n</i> (%)	841 (24.0)	103 (23.0)	0.09

BMI: Body mass index; SBP: Systolic blood pressure; DBP: Diastolic blood pressure; HDL-C: High-density-lipoprotein cholesterol; LDL-C: Low-density-lipoprotein cholesterol; FPG: Fasting plasma glucose; IFG: Impaired fasting glucose; IGT: Impaired glucose tolerance.

rum LDL-C, prevalence of abdominal obesity, low serum HDL-C and metabolic syndrome declined progressively with increased alcohol consumption among the three drinker groups (Table 4). When adjusted for sex and age, alcohol consumption still showed its effects on metabolic disorders among alcoholic drinkers.

DISCUSSION

In the present study, the sex- and age-adjusted prevalence of current alcohol drinking and metabolic syndrome among Shanghai adults was 13.0% and 15.3%, respectively; serum LDL-C concentration, risk of abdominal obesity, low serum HDL-C and metabolic syndrome tended to decrease in parallel to the history of alcohol consumption; arterial blood pressure, FPG and serum triglyceride concentration tended to increase in parallel to the amount of alcohol consumption; the effects of current alcohol drinking on metabolic syndrome features were independent of gender and age.

Metabolic syndrome is highly prevalent in industrialized Shanghai with a high Western life style^[15-17]. However, the prevalence of current alcohol drinking among Shanghai adults is relative lower (only accounting for 13.0% of the total population and 24.5% of men), than that in other cities of China (accounting for 27.0% in Hangzhou, Zhejiang Province and 35.1% in Xi'an, Shaanxi Province)^[18,19]. According to the Third National Health and Nutrition Examination Survey conducted in the USA, 57.9% of the participants were current alcohol drinkers with a higher percentage of men (66.0%) than of women (50.0%)^[9], and the prevalence of current alcohol drinking in 27030 healthy Korean men was even up to 83.3%^[20]. The majority of subjects were light to moderate alcohol drinkers, and the average daily alcohol consumption even in the exces-

Table 4 Effects of alcohol consumption on metabolic syndrome features in 448 subjects with current drinking according to the amount of alcohol consumption

	Light drinking (<i>n</i> = 180)	Moderate drinking (<i>n</i> = 143)	Excessive drinking (<i>n</i> = 125)	<i>P</i> value
BMI (kg/m ²)	24.9 ± 3.25	24.7 ± 4.59	24.9 ± 3.72	> 0.05
Waist-to-hip ratio	0.85 ± 0.12	0.86 ± 0.14	0.84 ± 0.18	> 0.05
SBP (mmHg)	132.3 ± 16.6	132.6 ± 17.1	133.7 ± 18.3	< 0.05
DBP (mmHg)	85.7 ± 10.1	85.4 ± 9.4	86.5 ± 12.0	> 0.05
Triglyceride (mmol/L)	1.47 ± 1.12	1.77 ± 1.61	1.66 ± 1.41	> 0.05
TC (mmol/L)	4.92 ± 0.92	4.86 ± 0.92	4.96 ± 0.94	> 0.05
HDL-C (mmol/L)	1.56 ± 0.39	1.57 ± 0.39	1.60 ± 0.44	< 0.05
LDL-C (mmol/L)	2.74 ± 0.79	2.72 ± 0.77	2.71 ± 0.92	< 0.05
FPG (mmol/L)	5.92 ± 1.46	5.96 ± 1.60	6.09 ± 2.10	< 0.01
Obesity, <i>n</i> (%)	84 (46.7)	60 (42.0)	55 (44.0)	> 0.05
Abdominal obesity, <i>n</i> (%)	66 (36.7)	34 (23.8)	29 (23.2)	< 0.05
Hypertension, <i>n</i> (%)	100 (55.6)	73 (51.1)	73 (58.4)	> 0.05
Hypertriglyceridemia, <i>n</i> (%)	53 (29.4)	46 (32.2)	41 (32.8)	< 0.05
Low HDL-C, <i>n</i> (%)	36 (20.0)	24 (16.8)	12 (9.6)	< 0.01
IFG/IGT, <i>n</i> (%)	43 (23.9)	43 (30.1)	32 (25.6)	> 0.05
Diabetes mellitus, <i>n</i> (%)	21 (11.7)	1 (0.70)	10 (8.0)	> 0.05
Metabolic syndrome, <i>n</i> (%)	46 (25.6)	32 (22.4)	25 (20.0)	< 0.05

BMI: Body mass index; SBP: Systolic blood pressure; DBP: Diastolic blood pressure; HDL-C: High-density-lipoprotein cholesterol; LDL-C: Low-density-lipoprotein cholesterol; FPG: Fasting plasma glucose; IFG: Impaired fasting glucose; IGT: Impaired glucose tolerance. *P* value for trend analysis reflects the overall difference among the three groups.

sive drinkers was less than 60 g/d in the present study. This might be related to the different alcohol drinking culture backgrounds worldwide and the universal concern about the overall health among Shanghai adults^[16,18,21]. The low prevalence of current alcohol drinking is consequently consistent with the uncommon alcoholic fatty liver (0.79%). Alcoholism only accounts for 5% of the etiology of fatty liver among Shanghai adults^[16].

Although alcohol consumption has a negative effect on the morbidity and mortality of liver disease, the correlation between alcohol consumption and metabolic syndrome gives rise to much controversy^[1,2,5,6,20,22-27]. A cross-sectional analysis of data from the Third National Health and Nutrition Examination Survey conducted in the USA showed that mild to moderate alcohol consumption is associated with a lower prevalence of metabolic syndrome, showing a favorable influence on lipids, waist circumference, and fasting insulin^[8,9]. Yoon *et al*^[24] reported that 1-15 g of alcohol per day is associated with decreased prevalence of metabolic syndrome. Park *et al*^[25] reported that the prevalence of metabolic syndrome decreases only in women, and Santos *et al*^[26] have not found any association between alcohol consumption and metabolic syndrome in both genders. However, Yokoyama *et al*^[22] reported that alcohol consumption (> 20 g/d) is associated with an increased prevalence of metabolic syndrome in male Japanese. In the present study, alcohol consumption was associated with reduced prevalence of metabolic syndrome and excessive alcohol drinkers had the lowest prevalence of metabolic syndrome. However, no statistically significant difference was found between alcohol and non-alcohol

drinkers. Inconsistent results were also found between alcohol consumption and obesity. Some studies demonstrated that light to moderate alcohol drinking can reduce weight whereas non-alcohol and heavy alcohol drinking cannot reduce weight^[1,2,23,25]. In the present study, neither positive nor negative association was found between alcohol consumption and BMI and risk of obesity. However, a consistent association was observed between alcohol consumption and decreased prevalence of abdominal obesity. Most excessive alcohol drinkers consumed only a little higher than 30 g/d in our study, and might be moderate alcohol drinkers in some other studies^[1,2,18,20,24,28-30]. Therefore, it is not entirely clear how different levels of alcohol consumption affect the risk of obesity and metabolic syndrome.

Epidemiologic investigations showed that moderate alcohol consumption is associated with increased blood pressure^[1,29,30]. It was reported that SBP and diastolic blood pressure (DBP) are increased to 2.7 mmHg and 1.4 mmHg, respectively, after a period of sustained alcohol consumption^[1,29]. In the present study, SBP and DBP had an average increase of 3.78 mmHg and 4.06 mmHg, respectively, in current alcoholic drinkers. Furthermore, SBP increased gradually in alcohol drinkers. A meta-analysis of data showed that excessive alcohol consumption was associated with a higher blood pressure while a fall in blood pressure of 2-4 mmHg is associated with reduced alcohol consumption^[30].

It is well known that increased concentration of triglycerides and HDL-C is associated with increased alcohol consumption^[1,2]. Interestingly, alcohol consumption was negatively associated with serum LDL-C concentration and was the lowest in excessive alcohol drinkers in this study, which is consistent with the findings in Korean men^[20].

The relationship between alcohol consumption and glucose regulation is rather complex^[1,2]. A meta-analysis based largely on the prospective studies suggested that there is a U-shaped relationship between alcohol consumption and type 2 diabetes, and moderate alcohol drinkers have the lowest risk of developing type 2 diabetes^[31]. However, several cross-sectional evaluations of healthier population have reported higher fasting glucose concentrations and greater risk of diabetes associated with alcohol consumption^[31-34]. Paradoxically, alcohol consumption has also been found to be associated with lower insulin concentrations^[8,20]. In our study, alcohol consumption was positively associated with FPG but negatively with the risk of diabetes. However, no change was observed in IFG and IGT, suggesting that regular alcohol consumption might benefit to insulin sensitivity and improve insulin resistance. However, it is not entirely clear how different levels of alcohol consumption affect glucose homeostasis.

The strengths of our investigation include the use of a large sample from Shanghai adults with good quality control (thereby enhancing our generalization) and the evaluation of both serum lipids and oral glucose tolerance. However, there are several limitations that merit comment. First, given the cross-sectional design, we could not draw any causal inferences regarding the association of alco-

hol consumption with metabolic syndrome. Second, the data on alcohol consumption were based on self-report with the possibility of misclassification of exposure (e.g., under reporting). However, such bias, if non-differential, would be expected only to increase the amount of alcohol consumption associated with reduced prevalence of metabolic syndrome. Third, other factors such as smoking, physical activity, and type of beverages were not excluded by multivariable linear regression analysis, thus limiting our ability to give comment on the relation of alcohol consumption to the prevalence of metabolic syndrome^[1,2,35]. Prospective studies are therefore needed to confirm these findings and to assess the influence of alcohol drinking patterns and other possible factors on the association between alcohol consumption and metabolic syndrome.

In summary, current alcohol drinking is associated with a lower prevalence of metabolic syndrome and a favorable influence on serum lipids, waist circumference, and possibly type 2 diabetes mellitus. The clinical significance of these findings needs further investigation. It must be noted that alcohol consumption also causes hypertension, hypertriglyceridemia and hyperglyceridemia, constituting alcohol-related metabolic syndrome. The latest American Heart Association guidelines advise that people should not start alcohol drinking if they have not drunk it, because it is not possible to predict who will have a problem due to alcohol abuse^[35].

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COMMENTS

Background

Alcohol consumption is a double-edged sword to health. Light to moderate alcohol consumption is usually associated with a lower cardiovascular mortality and a reduced risk of developing type 2 diabetes mellitus. However, heavy or risky alcohol drinking is toxic to the heart and overall health, and is the third leading cause for premature death among Americans. Metabolic syndrome is associated with an increased risk of developing diabetes and cardiovascular disease, the favorable influence of alcohol consumption on selective components of metabolic syndrome has contributed to the possibility that alcohol consumption reduces the risk of metabolic syndrome in the adults of USA, Japan and South Korean. However, its putative effect on metabolic disorders has not been well evaluated in mainland China or oversea Chinese.

Research frontiers

It was recently reported that moderate alcohol drinking is associated with a lower prevalence of metabolic syndrome. Although studies showed statistically significant interactions between alcohol consumption and metabolic syndrome, integration of variables and homogeneity in definitions is required. Since alcohol-associated health problem is also influenced by ethnicity, it is thus needed to investigate the interactions in Chinese.

Innovations and breakthroughs

One of the major findings in the present study is the relative lower prevalence of current alcohol drinking (13%) in Shanghai than in other regions of China and most foreign countries. This might be due to the different alcohol drinking culture backgrounds and the general concern about the dangers of alcohol consumption to the overall health among Shanghai people. However, metabolic syndrome is common in Shanghai as compared to Japan and South Korea, and the prevalence

of metabolic syndrome among Shanghai adults is only slightly lower than that in USA, suggesting that alcohol drinking is not a major risk factor for metabolic syndrome in humans, even though current alcohol consumption is associated with a lower prevalence of metabolic syndrome irrespective of the average daily alcohol consumption in the present study. On the one hand, alcohol drinking reduces serum LDL-C concentration, risk of abdominal obesity and low HDL-C. On the other hand, alcohol consumption might increase the prevalence of hypertension, hypertriglyceridemia and hyperglycemia. These effects of alcohol consumption on metabolic disorders are consistent with the most other related or similar findings from other countries. The results of this study provide the epidemiological data on the correlation between alcohol consumption and metabolic syndrome in Chinese.

Applications

There is sufficient evidence that light to moderate alcohol consumption is associated with decreased risk of cardiovascular disease. Nevertheless, the effects of alcohol consumption on health are dependent on the consumed amount of alcohol, the pattern of drinking, and the potential for problem drinking, suggesting that alcohol should not be advised for health enhancement of individuals.

Terminology

Metabolic syndrome refers to a cluster of metabolic derangements that increased the risk of developing type 2 diabetes and cardiovascular diseases associated with insulin resistance. Current alcohol drinker refers to alcohol drinking habituation regardless of the amount of alcohol consumption, and is usually defined as more than once of alcohol drinking per month over the past 12 mo. The classification of current alcohol consumption is far from consensual at present. In the present study, light alcohol drinking is defined as average alcohol consumption of less than 10 g/d, moderate alcohol drinking as average daily alcohol consumption of 10-30 g/d, and excessive alcohol drinking as average alcohol consumption of equal to or more than 30 g per day in the past 12 mo.

Peer review

The authors conducted a population-based study on 3953 adults in Shanghai to evaluate the association between alcohol consumption and metabolic syndrome, and found that current alcohol consumption was associated with a lower prevalence of metabolic syndrome. Overall, the study was well designed with good quality control, and detailed data were collected from participants with a randomized multistage stratified cluster sampling method. The manuscript provides important data and is relatively well written.

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Role of the duodenum in regulation of plasma ghrelin levels and body mass index after subtotal gastrectomy

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by 3.3% and 6.4%, respectively. The linear regression correlations were revealed in both groups between decrease of ghrelin level and BMI ($R_1^2 = 0.297$, $P = 0.007$; $R_2^2 = 0.559$, $P < 0.001$).

CONCLUSION: Anatomically and physiologically, the duodenum compensatively promotes ghrelin recovery and accordingly enhances BMI after gastrectomy. Regarding patients with insufficient ghrelin secretion, ghrelin is positively associated with BMI.

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Key words: Duodenum; Ghrelin; Body mass index; Subtotal gastrectomy; Digestive reconstruction

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Abstract

AIM: To investigate the role of the duodenum in the regulation of plasma ghrelin levels and body mass index (BMI), and the correlation between them after subtotal gastrectomy.

METHODS: Forty-two patients with T₀₋₁N₀₋₁M₀ gastric cancer were divided into two groups after gastrectomy according to digestive reconstruction pattern, Billroth I group ($n = 23$) and Billroth II group ($n = 19$). Ghrelin levels were determined with radioimmunoassay (RIA) before and on d 1, 7, 30 and 360 after gastrectomy, and BMI was also measured.

RESULTS: The two groups had identical postoperative trends in ghrelin alterations during the early stage, both decreasing sharply to a nadir on d 1 (36.7% vs 35.7%), then markedly increasing on d 7 (51.0% vs 51.1%). On d 30, ghrelin levels in the Billroth I group were slightly higher than those in the Billroth II group. However, those of the Billroth I group recovered to 93.6% on d 360, which approached, although lower than, the preoperative levels, and no statistically significant difference was observed. Those of the Billroth II group recovered to only 81.6% and manifested significant discrepancy with preoperative levels ($P = 0.033$). Compared with preoperative levels, ghrelin levels of the two groups decreased by 6.9% and 18.4% and BMI fell

INTRODUCTION

Ghrelin, a recognized brain-gut peptide with 28 amino acids, was recently discovered as the intrinsic ligand of the secretagogue receptor^[1], which exerts considerable vital physiological activities within the body, beyond stimulating growth hormone release. The duodenum is the major source of ghrelin secretion apart from the stomach, which is responsible for most of ghrelin production in the body^[2], while the bowel and some organs secrete less through paracrine or autocrine mechanisms, which accounts for the partially compensatory secretion after gastrectomy^[3-8]. Known as a unique orexigenic hormone, ghrelin contributes to maintaining the body's energy balance by communicating signals of energy stores to the brain, then inducing appetite, promoting food intake, and reducing energy expenditure^[5-9].

Gastrectomy is the predominant therapy for most stomach-associated diseases, including tumors and complex ulcers. However, weight loss is a ubiquitous sequela for which no satisfactory explanations have been

given, although various measures have been employed to treat it, including drugs, diet therapy, and even secondary surgery. Regarding the gastroduodenum as the main source of ghrelin, which has a considerable influence on energy balance, a lot of research has focused on the correlation between ghrelin and energy balance. However, to date, there have been no studies on the role of the duodenum in regulating ghrelin plasma levels and body mass index (BMI) after gastrectomy. It is thought that the digestive endocrine and exocrine functions will be impaired if the duodenum is devoid of long-term contact with gastric contents^[8,10-12]. Accordingly, we hypothesize that a similar situation would exert the same influence on ghrelin levels.

MATERIALS AND METHODS

Study subjects

Patients with T₀₋₁N₀₋₁M₀ (AJCC, American joint committee on cancer, 2003) stage gastric cancer were enrolled in our study from September 2004 to July 2006. Their clinical characteristics were collected, comprising gender, age, height, weight and BMI. Patients with coincidental endocrine diseases such as diabetes mellitus, thyroid or pituitary disease were excluded, as were those with BMI beyond the range of 18-26 kg/m². Curative subtotal gastrectomy, defined as resection of no less than two-thirds of the distal stomach, with standard D₂ lymph node resection, was performed on each patient by the same team. Those who contracted severe complications, such as anastomosis leakage, intra-abdominal infection, or ileus, and those who displayed tumor recurrence or metastasis by endoscopy, abdominal computer tomography (CT), sonography, or tumor biomarkers during regular follow-up, were also excluded. The subjects were divided into two groups according to their reconstruction patterns of digestive continuity, gastroduodenal anastomosis, i.e., Billroth I anastomosis ($n = 23$); and gastrojejunal anastomosis, with an end-to-side anastomosis of the jejunum, i.e., Billroth II anastomosis ($n = 19$).

As concerns the impact of the operation itself on ghrelin levels, during the same period, we chose 20 colorectal cancer patients who underwent surgery in our department as a control group, with the same screening conditions as the study group. All patients gave their informed consent before the study. Blood samples (10 mL each) were collected at 8:00 a.m. preprandially before and on d 1, 7, 30 (1 mo) and 360 (1 year) after operation. All patients fasted from midnight before blood collection. Plasma ghrelin and leptin levels of each sample were analyzed. In addition, with regard to the impact of cancer on both hormones, subjects were contrasted with 20 healthy individuals whose blood samples were drawn during regular physical examinations.

Hormone measurements

All blood samples were drawn into tubes containing EDTA and aprotinin. Plasma was obtained and stored at -70°C until assayed. Blood samples were centrifuged at 4°C for 15 min. Total plasma ghrelin was measured with a commercially available RIA kit (Phoenix Pharmaceuticals,

Belmont, CA, USA) using ¹²⁵I-labelled ghrelin tracer and a rabbit polyclonal antibody against full-length octanoylated human ghrelin that measures total circulating ghrelin. Plasma leptin was measured with a human RIA kit (Linco Research, Inc., St. Charles, MO, USA) using ¹²⁵I-labelled leptin tracer. Both hormones were measured in duplicate and the mean was determined.

Statistical analysis

All results were expressed as mean \pm SD. ANOVA with Student-Neuman-Keul post hoc test was used to determine the statistical significance of differences in ghrelin, leptin levels and BMI between the two groups before operation, and ghrelin or leptin levels of the same group between different time points after operation. Differences of ghrelin levels between the two study groups at the same time after operation were analyzed with the unpaired *t* test. Both decrease and increase of ghrelin, leptin levels and BMI were expressed as a percentage of the preoperative levels. The relationship between them was displayed with linear regression. The coefficient of correlation R^2 was used as the gauge of their relationship. A one-tailed test was adopted in each analysis. $P < 0.05$ was considered statistically significant.

RESULTS

Influence of duodenum on plasma ghrelin level and BMI after gastrectomy

There was no significant difference in preoperative characteristics, including ghrelin, leptin levels and BMI between the groups (Table 1). As for the control group, ghrelin levels increased slightly at first, then gradually decreased to preoperative levels during 360 d after operation (Figure 1). Leptin levels of all groups were less affected and no difference existed between the groups at the same time. The two groups had identical trends in ghrelin levels during the early stage after the operation, decreasing sharply to a nadir on d 1 (36.7% *vs* 35.7%). The levels then increased markedly on d 7 (51.0% *vs* 51.1%), and showed no difference between the two groups at the same time (169.35 ± 45.9 pg/mL *vs* 163.7 ± 49.3 pg/mL; 235.4 ± 61.3 pg/mL *vs* 232.1 ± 67.0 pg/mL). Nevertheless, the ghrelin levels of the Billroth I group recovered more obviously during the later stage compared with the Billroth II group (Figure 2). On d 30, ghrelin levels in the two groups increased to 70.6% (330.2 ± 77.1 pg/mL) and 67.2% (300.3 ± 80.1 pg/mL) respectively, with those of the Billroth I group higher than those of the Billroth II group, whereas no significant difference existed between the two. On d 360, ghrelin levels of the Billroth I group recovered to 93.1% (435.9 ± 110.2 pg/mL), although they were lower than preoperative levels, but no significant difference was revealed. However, ghrelin levels in the Billroth II group recovered to only 81.6% (369.7 ± 90.1 pg/mL), evidently lower than preoperative levels ($P = 0.033$). On d 360, ghrelin levels in the Billroth I group were distinctly higher than those in the Billroth II group ($P = 0.035$). From d 7 through to d 360, ghrelin levels in the two groups increased by 42.1% and 30.6%, respectively, with a significant difference between the levels ($P = 0.003$).

Table 1 Preoperative characteristics of the study objects

Group	Healthy	Control	Billroth I	Billroth II
Object number	20	20	23	19
Gender (male:female)	8:12	13:7	11:12	8:11
Age (yr)	40.4 ± 10.2	42.2 ± 7.4	50.7 ± 5.5	48.0 ± 6.4
BMI	22.2 ± 2.2	22.7 ± 2.2	21.9 ± 2.8	21.7 ± 2.7
Ghrelin (pg/mL)	460 ± 117.8	472 ± 115.9	468.0 ± 126.9	460.5 ± 129.4
Leptin (ng/mol)	2.0 ± 1.2	2.4 ± 1.1	2.2 ± 1.3	2.6 ± 1.7

Correlation between ghrelin and BMI after gastrectomy

Compared with preoperative levels, ghrelin levels in the Billroth I and Billroth II groups decreased by 6.9% and 18.4%, respectively, on d 360, which showed a significant difference between the two groups ($P = 0.035$), while BMI decreased by 3.3% and 6.4%, respectively, which also showed a significant difference ($P = 0.035$). The linear regression correlations were manifested between decrease of ghrelin level and BMI in both groups ($R_1^2 = 0.297$, $P = 0.007$; $R_2^2 = 0.559$, $P < 0.001$). Neither the correlation nor the regression coefficient of the Billroth II group was higher than that of the Billroth I group (Figure 3).

DISCUSSION

Ghrelin has recently been implicated in the development of malignant tumors^[13], whereas these tumors did not show any effect on plasma ghrelin levels in our study. Postoperative ghrelin levels in colorectal cancer patients increased and then recovered gradually to normal, while those in gastric cancer patients decreased abruptly in the early stage, then rose gradually, although remaining lower, one year later compared with the preoperative level. We can generalize from the above that a postoperative decrease in ghrelin was induced by gastrectomy, but not the surgery itself or by trauma, or anesthesia or any factors involved in anesthesia.

As far as gastrectomy was concerned, a major portion of the stomach was removed, including part of the fundus, a major source of ghrelin production. This accounted for a sharp postoperative decrease in ghrelin levels, whereas due to compensatory secretion of the remaining part of the stomach, the duodenum, and other organs involved, ghrelin levels increased gradually.

Intriguingly, in the early phase after the operation, the two groups manifested identical profiles of ghrelin levels, whereas in the late phase, the ghrelin levels in the Billroth I group increased more distinctly than those in the Billroth II group, although neither approached preoperative levels. We consider that the cause of this was the discrepant restoration patterns of digestive continuity. This reveals that the duodenum is the most crucial ghrelin-producing organ, except for the stomach. The duodenum compensatively secretes ghrelin more effectively in an anatomical-physiological continuity, as in a Billroth I anastomosis, as compared to that in the relatively isolated Billroth II anastomosis.

This implies that long-term absence of contact with gastric contents exerts a predominant effect on the duodenum in postoperative ghrelin secretion. It is

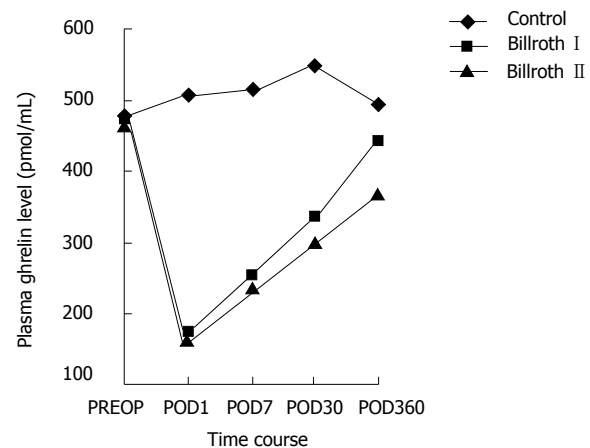


Figure 1 Alterations of ghrelin level during 360 d after operation of the two study groups and the control group. POD: Postoperative day.

considered by some researchers that gastroduodenal exposure to food is not essential to ghrelin secretion, in that most X/A-like cells are closed, which means that they have an impact upon the basolateral membrane adjacent to the bloodstream, but do not open towards the digestive lumen^[2,14]. However, merely by virtue of this, it cannot be denied that there is probably a trigger for the production of ghrelin related to food contact by some other unknown means. Furthermore, X/A-like cells in the duodenum and intestine gradually open concurrently towards the lumen and the microvascular circulation.

Compatible with our point, Qader *et al* have demonstrated ghrelin concentrations in plasma and gastroduodenal mucosa in rats given short-term total parental nutrition were reduced by 50% simultaneously^[15]. Long-term lack of food contact would reduce the duodenum to atrophy and hypoplasia, and even disturb endocrine functions, which if severe, would lead to pathophysiological disorders^[16,17]. Thus, anatomical-physiological normalcy is essential for the duodenum to exert its customary ghrelin-producing activity after gastrectomy.

It has been revealed that ghrelin levels decrease as biliopancreatic limbs lengthen in Roux-en-Y gastric bypass surgery for obesity^[18], which is in accordance with our view that the opportunity for interactive contact between the duodenum and gastric contents decreases as biliopancreatic limbs lengthen. Cummings *et al* have demonstrated downregulation or reduction of ghrelin levels may be a result of "overridden inhibition" of gastroduodenal ghrelin-producing cells isolated from contact with enteral nutrients. This resembles the paradoxical suppression of growth hormone by continuous signaling from gonadotropin-releasing hormone or growth hormone-releasing hormone^[19,20]. However, it lacks convincing proof and further investigation is required to decipher how the duodenum regulates ghrelin production and levels after gastrectomy with different digestive continuity reconstructions.

Considerable evidence supports the view that ghrelin levels correlate inversely with BMI and manifest compensatory changes in response to body weight

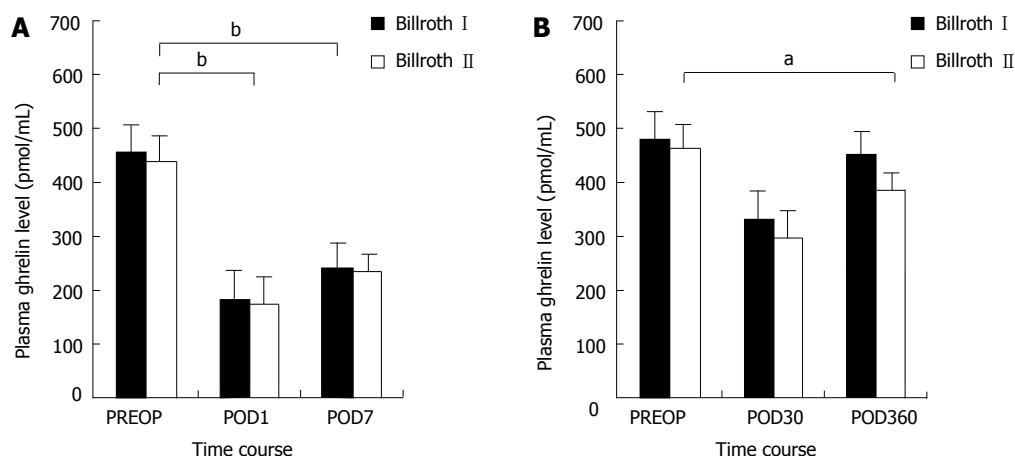


Figure 2 Alterations of ghrelin levels of two study groups at the early (A) and later (B) stage. ^a $P < 0.05$, ^b $P < 0.01$.

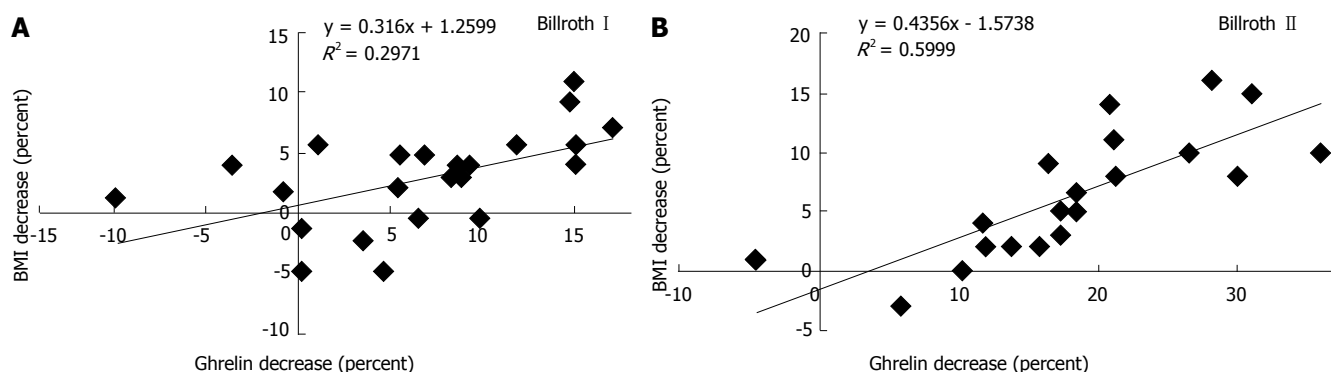


Figure 3 Linear regression correlations. (A) Detected between decrease in ghrelin level and BMI in Billroth I group patients 1 yr after operation. (B) Detected between decrease in ghrelin level and BMI in Billroth II group patients 1 yr after operation. $R^2 = 0.297$, $P < 0.007$. $R^2 = 0.559$, $P < 0.001$.

alterations^[20-25]. Several clinical studies have demonstrated long-term administration of ghrelin promotes weight gain in patients with cachexia due to chronic heart failure, chronic obstructive pulmonary disease, or even malignant tumors, by stimulating food intake, decreasing energy expenditure and regulating other aspects of energy homeostasis^[21,25,26].

As regards patients with insufficient ghrelin secretion after gastrectomy, we found it interesting that a decrease in ghrelin level was positively correlated with a decrease in BMI. This means that the ghrelin level is positively associated with BMI in individual patients, which implies that postoperative weight loss results from down-regulation of ghrelin levels after gastrectomy, and may be treated with administration of exogenous ghrelin. With regard to linear regression correlation between ghrelin levels and BMI, the Billroth II group presented a higher correlation or regression coefficient compared with the Billroth I group, which implies that the efficacy of ghrelin for the regulation of BMI is associated to some extent with its concentration.

In summary, anatomical-physiological duodenal normalcy promotes ghrelin recovery through compensatory secretion, thereby leading to BMI recovery, while an isolated duodenum displays obviously insufficient ghrelin secretion, which results in a decrease in BMI. Regarding the individual patient with insufficient ghrelin secretion after gastrectomy, ghrelin levels correlate positively with BMI.

COMMENTS

Background

It has been revealed that ghrelin contributes to weight gain and maintaining energy balance within the body, and weight loss is a ubiquitous sequela after subtotal gastrectomy. It is unclear whether anatomical-physiological duodenal normalcy promotes ghrelin recovery through compensatory secretion and weight gain after gastrectomy.

Research frontiers

The aim of this study was to analyze whether the duodenum plays a role in regulation of plasma ghrelin levels and body mass index (BMI) after subtotal gastrectomy.

Innovations and breakthroughs

This was a prospective clinical study that focused on the duodenum in different digestive reconstruction models, which contributed differently to ghrelin secretion and weight gain.

Applications

This study provides surgeons with evidence to apply digestive reconstruction that has an anatomical-physiological duodenal normalcy during operation, and it also reveals that exogenous ghrelin may be useful in therapy of severe weight loss due to gastrectomy.

Terminology

Ghrelin, a brain-gut peptide, was recently discovered as the intrinsic ligand of the secretagogue receptor. BMI (kg/m^2) is widely used as a gauge to measure fat storage within the body.

Peer review

This was a study of the long-term effect of exclusion of the duodenum on the

production of ghrelin, and presents original information about the role of the duodenum. It is very interesting.

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S- Editor Li DL L- Editor Kerr C E- Editor Lu W



RAPID COMMUNICATION

Study on vasculogenic mimicry in malignant esophageal stromal tumors

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Abstract

AIM: To investigate whether malignant esophageal stromal tumors contain PAS-positive patterned matrix-associated vascular channels, which are lined by tumor cells, but not vascular endothelial cells. That is vasculogenic mimicry (VM) independent of tumor angiogenesis.

METHODS: Thirty-six tissue samples of malignant esophageal stromal tumors were analyzed. Tissue sections were stained for Vascular endothelial growth factor (VEGF), CD31 and periodic acid Schiff (PAS). The level of VEGF, the microvascular density (MVD) and the vasculogenic mimicry density (VMD) were determined.

RESULTS: PAS-positive patterned matrix-associated vascular channels were detected in 33.3% (12/36) of tumor samples. Within these patterned channels, red blood cells were found. The level of VEGF and the MVD in tumors containing patterned channels were significantly higher than those in tumors not containing patterned channels ($P < 0.05$). At the same time, the malignant degree of tumors was higher, the proportions of tumors containing patterned channels were not only more, but also in the each kind of tumors containing patterned channels.

CONCLUSION: In malignant esophageal stromal tumors, a VM mechanism causes some tumor cells to deform themselves and secrete extracellular matrix; thus, PAS-positive patterned matrix-associated vascular channels appear and supplying blood to the tumors to sustain their growth and metastasis.

INTRODUCTION

Vasculogenic mimicry is defined by the phenomenon whereby plastic malignant tumor cells transform and excrete extracellular matrix, thus mimicking the blood transport channel formation of normal vascular tissues^[1]. Vasculogenic mimicry (VM) is a totally new pattern of tumor angiogenesis, which differs from normal angiogenesis remarkably. Tumors containing VM show such biological behaviors as higher malignancy, non-directional or bi-directional, rapid proliferation, and high incidence of metastasis by a vascular route^[2]. VM was first discovered in a human uvea malignant melanoma by Maniotis^[1] in 1999. In recent years, it has been reported that VM mainly exists in bi-directional malignant tumors, such as malignant melanomas in other sites, synovial sarcomas, mesotheliosarcomas, sarcoma epithelioides and acinus rhabdomyosarcomas^[2]; it was also found in inflammatory breast cancers, inflammatory liver cancers, prostate cancers and ovary cancers^[3]. Based on the features of VM discussed above, with regard to the fact gastrointestinal stromal tumors are a group of non-directional tumors, commonly vascular stromal tumors arising from the gastrointestinal mesenchymal cells (Cajal cells), we selected esophageal stromal tumors to investigate the existence of VM. To date, similar studies on the relationship between VM and malignant esophageal stromal tumors have not been reported broadly.

MATERIALS AND METHODS

Patients

Among paraffin samples collected from 1997 to 2000

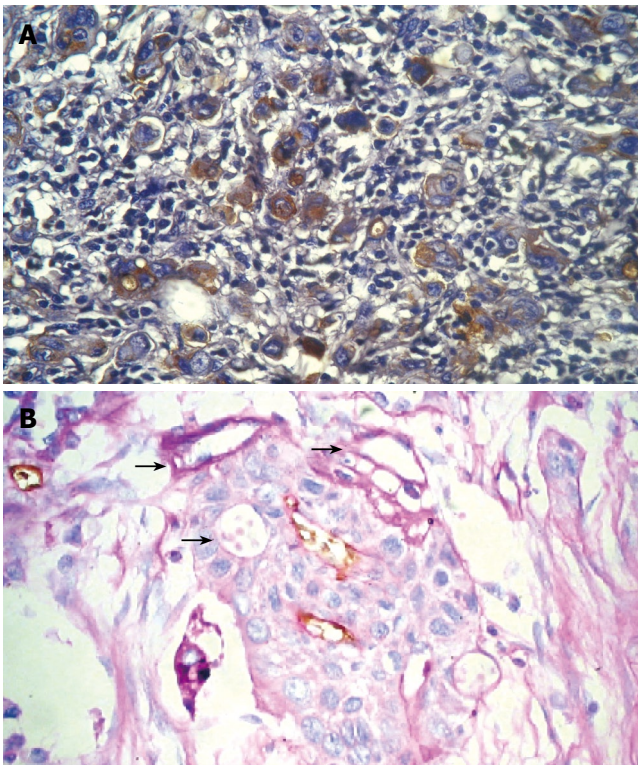


Figure 1 A: VEGF and PAS double staining; B: CD31 and PAS double staining ($\times 400$).

in Shandong University, Qilu Hospital, 36 samples of malignant esophageal stromal tumors with follow-up documents were selected and observed. Eighteen of these showed low malignancy, while the others were highly malignant. Combined with a review of the history, they were diagnosed once again and stratified into low malignancy and high malignancy groups based on the degree of differentiation and mitosis count, and the existence of necrosis.

Immunohistochemistry

At least 4 sections were obtained from each sample, and these were stained for VEGF, CD31, and PAS by immunohistochemistry as described previously^[2]. The glass slides were silicified, and the cover slides were soaked and washed with 50 mL of 75% ethanol containing one drop of concentrated hydrochloric acid.

The streptavidin peroxidase method was used. Mouse monoclonal anti-human VEGF and CD31 antibodies, caprine-anti-mouse IgG and DAB developer were all purchased from Zhongshan BioTechnologies Company. The periodic acid Schiff (PAS) reagents were prepared in our laboratory. Three samples of gastric mucous adenocarcinomas were stained as a control group. All of the mucous locations were stained cherry red, which proved the reliability of the quality of the PAS reagents.

The level of VEGF was evaluated using a stereological grid counting method. CD31- and PAS-stained sections were observed using a 200 times objective on a light microscope; the positive CD31 and PAS staining images were taken as measurement objects. Ten fields of vision were selected randomly to assess the microvascular density

Table 1 Relationship between VM and the expression of VEGF and MVD

Existence of VM	Samples	VEGF	MVD
Yes	12	91.49 \pm 29.12	45.84 \pm 13.81
No	24	128.39 \pm 18.45	76.92 \pm 14.62
<i>t</i> value		3.874	5.972
<i>P</i> value		0.002	0

(MVD) and the vasculogenic mimicry density (VMD); the average of the 10 counts was taken as the ultimate expression of MVD and VMD.

Statistical analysis

Statistical analyses were performed using software from SPSS for Windows 10.0. The data was analyzed with a matched paired rank sum test. $P < 0.05$ was taken to indicate statistical significance.

RESULTS

The distribution of VM in malignant esophageal stromal tumors

Based on CD31 and PAS staining, CD31-negative, PAS-positive vascular-like patterns, which indicated VM, could be seen in 33.3% (12/36) of malignant esophageal stromal tumors. VM could be found in 20.0% (4/20) of low malignancy tumors and 50.0% (8/16) of highly malignant tumors; this difference in the level of VM was statistically significant. Analysis of clinical data revealed that the 5 years survival rate of tumors expressing VM was 8.3 % (1/12), which is much less than that of tumors not expressing VM (41.7%; 10/24).

PAS-positive malignant esophageal stromal tumors

The PAS-positive human uvea melanomas identified by Maniotis showed linear, parallel linear, cruciform, half-moon, annuliform, and lattice forms^[1]. All of the forms talked about above were also found in malignant esophageal stromal tumors. Besides, red blood cells were also found in the angiogenesis mimicry (Figure 1).

The levels of VEGF as well as MVD in tumors including VM were less than those in tumors that did not include VM ($P = 0.002$, $P < 0.0001$, respectively; Table 1). With increased tumor malignancy, the levels of VEGF, as well as MVD and VMD increased gradually, all of which showed statistical significance between high and low malignancy tumors ($P = 0.047$, $P = 0.002$, $P = 0.037$, respectively). Moreover, among low malignancy tumors, MVD was greater than VMD. However, the opposite was true of highly malignant tumors (Table 2).

DISCUSSION

Maniotis *et al* firstly reported the phenomenon of VM in 1999, providing a primary description and explanation. They found in human uvea malignant melanoma new blood transport channels, the walls of which were formed by metamorphoses from the malignant melanoma and its matrix. Because the structure of these channels was

Table 2 Relationship between the degree of tumors' malignancy and the expression of VEGF, as well as MVD and VMD

Malignancy	Samples	VEGF	MVD	VMD
Low	4	45 ± 19	15 ± 8	38 ± 25
High	8	128 ± 42	81 ± 17	122 ± 39
F value		4.1262	12.139	5.826
P value		0.047	0.002	0.037

similar to that of the normal vasculature, it was called angiogenesis mimicry. VM channels, co-existing with the normal vasculature, formed the microcirculation system concomitantly in tumors, and provided blood supply for their growth. Light microscopic, electron microscopic and immunohistochemical investigations of endothelial cells confirmed that the walls of VM channels consisted of tumor cells, not vascular endothelial cells. However, neither necrosis nor fibrosis was found in areas containing VM channels. PAS staining showed that a layer of PAS-positive basal membrane was found outside of the VM channels, while confocal laser scanning microscopy revealed that red blood cells could be seen in VM channels. then they found in tumor cells three dimensional cultures vascular reticulate structure, reproduced the former discovery and confirmed the blood cells in VM channels. Taking advantage of macromolecular contrast agents, dynamic MRI angiography revealed VM channels communicating with the normal vasculature in inflammatory breast cancers^[3]. The blood flow was also found based on such technique. Genetic array analysis showed that highly invasive melanoma cells showed plasticity and a polyergic embryoid genetic phenotype; thus, they could mimic endothelial cells, forming VM channels that were strikingly different from normal vascular channels. VM channels had the features of embryoid invasion, which were commonly found in invasive tumors and related to the invasion and prognosis of tumors^[5].

Our study selected 36 malignant esophageal stromal tumors. Based on CD31 and PAS staining, VM was observed. With increasing malignancy of tumors, the proportion of VM tumors increased gradually; the level of VEGF, as well as MVD and VMD also increased gradually, showing positive correlation tendency with the presence of VM. The possible causes of this are as follows. The higher the malignancy, the more rapidly tumor cells divide and proliferate, the more immature their differentiation, and thus, the plasticity they show. If tumors grow too rapidly, the current vascular would not be able to provide sufficient nutrition and oxygen; thus, hypoxia would induce the expression of VEGF and further tumor angiogenesis, concomitant with the high expression of CD31 or MVD. If the tumor angiogenesis still could not synchronize with the rapid growth of tumors, partial tumor cells may then deform and excrete extracellular matrix, mimicking the normal vascular structure, forming a blood transport system, and communicating with the normal vasculature, all of which ensure the blood supply to new tumor cells^[5]. At the same time, it could also be appreciated that when angiogenesis did not synchronize with the growth of tumors and VM is not found in some part of malignant

gastrointestinal stromal tumors, necrosis or fibrosis is an inevitable event^[1-5]. Our study also observed that, on the inner walls of a minority of VM channels, CD31 was expressed; this could be explained by the assumptions that few tumor cells on the inner walls of VM channels have potent plasticity, could mimic the activity of endothelial cells, and have the capacity to excreting CD31 molecules. However, it remains to be determined whether the VM phenomenon is just an emergent mechanism when the tumor cells are in the "condition of hunger", which serves to solve the shortage of blood supply resulting from the rapid growth of tumor cells, temporarily, and is then replaced by normal vasculature formed by endothelial cells with the passage of time.

Analysis of clinical data revealed tumors that formed more VM channels were usually found in patients showing early metastasis, poor prognosis, and shorter life span. The formation of VM channels may be explained as follows. VM channels and normal vasculature form the tumor's microcirculation concomitantly and communicate with the body's circulation system; thus, the tumor becomes rich in vasculature. The inner walls of VM channels consist of metamorphosed tumor cells, which structures are crumbly than normal vascular, all of which serve to the tumor cells' blood route metastasis^[6]. Recent research^[7] showed more highly invasive malignant melanomas express more protein tyrosine kinase (PTK), and immunofluorescence showed that protein tyrosine kinase phosphorylation was centered specifically in the area where VM channels formed. Yet the PTK signal transduction path has a close relationship with various intracellular growth factors, overexpression of which could facilitate the growth, differentiation, proliferation, adhesion and metastasis of tumor cells. Thus, it could be concluded PTK overexpression has a close relationship with the formation of VM channels and the blood metastasis of tumor cells. That study also confirmed the capacity of inhibitors of PTK to decrease VM in malignant tumors. Currently, the inhibitor of PTK imatinib has been extensively used in the clinical treatment of gastrointestinal stromal tumors, primarily being targeted toward inhibiting continuous activation of Kit PTK caused by the mutation of oncogene *c-Kit* in GIST, thus further decreasing the division and proliferation of tumor cells^[8]. However, the capacity of inhibitors of PTK to decrease VM could be one of the reasons why such drugs could be successfully used in the treatment of GIST. It was also reported^[9] that MMP (matrix metalloproteinase) inhibitor, PI-3K (phosphatidylinositol-3 kinase) inhibitor, PSMA (prostate-specific membrane antigen) inhibitor, tetrocycalamycin (CMT-3) modified chemically, and an antibody against laminin or laminin 5γ2 strand antisense oligonucleotides have a role in the inhibition of VM.

The phenomenon of VM deepens our knowledge about the mechanisms underlying tumors' blood supply and blood metastasis. However, study of VM in tumors is still at the early stage, and further research and discussion on various problems is required. It is reasonable to believe that with increased knowledge of its underlying mechanism, inhibiting VM is sure to provide a new target in the treatment of various neoplasms, including malignant esophageal stromal tumors.

COMMENTS

Background

Many studies have declared vasculogenic mimicry as a new pattern of tumor angiogenesis, which differs from the normal angiogenesis remarkably. Studies on the relationship between vasculogenic mimicry (VM) and malignant esophageal stromal tumors have not been reported broadly.

Research frontiers

This study was designed to investigate whether malignant esophageal stromal tumors contain PAS-positive patterned matrix-associated vascular channels.

Innovations and breakthroughs

In malignant esophageal stromal tumors, VM enables tumors to establish additional blood supply to sustain their growth and metastasis

Peer review

The authors investigated the relationship of VM and malignant esophageal stromal tumors. It is a very interesting study.

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RAPID COMMUNICATION

Impact of postoperative omega-3 fatty acid-supplemented parenteral nutrition on clinical outcomes and immunomodulations in colorectal cancer patients

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Abstract

AIM: To investigate the effect of omega-3 fatty acid parenteral supplementation postoperatively on clinical outcomes and immunomodulation in colorectal cancer patients.

METHODS: Forty-two patients undergoing radical colorectal cancer resection with an indication for total parenteral nutrition postoperatively were enrolled in this prospective, double-blind, randomized, controlled study. Patients received total parenteral nutrition supplemented with either soybean oil (LCT; Intralipid®, Fresenius-Kabi, SO group, $n = 21$) or a combination of omega-3 fish oil and soybean oil (LCT:fish oil = 5:1, fish oil; Omegaven®, Fresenius-Kabi, FO group, $n = 21$), up to a total of 1.2 g lipid/kg per day for 7 d postoperatively. A same volume calorie and nitrogen was administrated. Routine blood test, biochemistry, systemic levels of IL-6 and TNF- α , percentage of CD3⁺, CD4⁺, and CD8⁺ lymphocytes were evaluated preoperatively and on postoperative d 1 and 8. Patient outcome was evaluated considering mortality during the hospital stay, length of postoperative hospital stay, and occurrence of infectious complications.

RESULTS: Both lipid regimens were well tolerated. No differences between the two groups were noticed in demographics, baseline blood test, biochemistry, serum levels of IL-6 and TNF- α , percentage of CD4⁺, CD8⁺ lymphocytes, and ratios of CD4⁺/CD8⁺. Compared with those on postoperative d 1, serum IL-6 levels on

postoperative d 8 were significantly depressed in the FO group than in the reference group (-44.43 ± 30.53 vs -8.39 ± 69.08 , $P = 0.039$). Simultaneously, the ratios of CD4⁺/CD8⁺ were significantly increased in the FO group (0.92 ± 0.62 vs 0.25 ± 1.22 , $P = 0.035$). In addition, depression of serum TNF- α levels (-0.82 ± 2.71 vs 0.27 ± 1.67 , $P = 0.125$) and elevation of CD3⁺ and CD4⁺ lymphocyte percentage (12.85 ± 11.61 vs 3.84 ± 19.62 , $P = 0.081$, 17.80 ± 10.86 vs 9.66 ± 17.55 , $P = 0.084$, respectively) were higher in the FO group than in the reference group. Patients in the FO group trended to need a shorter postoperative hospital stay (17.45 ± 4.80 d vs 19.62 ± 5.59 d, $P = 0.19$). No statistically significant difference was found when stratified to mortality and occurrence of infectious complications.

CONCLUSION: Postoperative supplementation of omega-3 fatty acids may have a favorable effect on the outcomes in colorectal cancer patients undergoing radical resection by lowering the magnitude of inflammatory responses and modulating the immune response.

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Key words: Colorectal cancer; Parenteral nutrition; Omega-3 fatty acids; Immunomodulation; Abdominal surgery

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INTRODUCTION

Lipid emulsions are regularly used postoperatively to supply energy and essential fatty acids^[1]. Recently, the pharmacological role of fatty acid and omega-3

polyunsaturated fatty acid (PUFA) deficiency in colorectal cancer patients has been appreciated^[2]. Conventional lipid soybean oil emulsions contain a very large amount of linoleic acid (LA; 18: 2n-6) and a relatively low amount of α -linolenic acid (LNA; 18: 3n-3). Arachidonic acid (AA, C20: 4n-6), derived from linoleic acid, is metabolized by cyclo-oxygenase and lipo-oxygenase pathway to pro-inflammatory mediators, such as prostaglandin, thromboxane, and leukotriene. Omega-3 long-chain polyunsaturated fatty acid, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), which can compete with AA for the production of less inflammatory active eicosanoid, are absent in these vegetable oils^[3,4]. Therefore, omega-3 fatty acids should be added directly into lipid emulsions to suppress the system inflammatory response and to decrease the risk of postoperative thrombosis. Recently, supplementation with fish oil is supposed to improve standard clinical therapy for chronic hyper-inflammatory diseases such as Crohn's disease^[1,5], rheumatoid arthritis^[6], cancer cachexia^[7,8], and as an adjunct therapeutic measure for trauma, injury, and sepsis^[2,9-12]. Although several studies have demonstrated the beneficial effects of omega-3 fatty acid supplementation on patient outcome or immune competence, randomized controlled clinical trials focusing on the use of parenteral fish oil are scarce^[13-17]. The aim of this study was to assess whether parenteral supplementation of omega-3 fatty acid postoperatively improves the inflammatory and immunological function of colorectal cancer patients and their clinical outcomes.

MATERIALS AND METHODS

Patients

Forty-two patients with colon or rectal cancer staging TNM I-III undergoing radical resection, who gave their written, informed consent to participate in the study, were prospectively enrolled consecutively from May 2002 to October 2003. After operation, 41 patients were randomly assigned to receive total parenteral nutrition (TPN) supplemented with either soybean oil (SO) or SO + fish oil (FO) emulsion, one patient withdrew because of the unresectable disease. The clinical characteristics of the two groups of patients are summarized in Table 1.

Exclusion criteria

Exclusion criteria were: (1) age < 18 or > 70 years; (2) body mass index (BMI) < 16 or > 30; (3) diabetes mellitus; (4) hypertriglyceridemia (> 200 mg/dL) or hypercholesterolemia (> 240 mg/dL); (5) abnormal liver function (ALT > 60 IU/L or total bilirubin > 1.2 mg/dL); (6) abnormal renal function (serum creatinine > 1.6 mg/dL or BUN > 30 mg/dL); (7) post-splenectomy; (8) endocrine diseases, such as hyperthyroidism, hyperadrenocorticism, or medication with thyroxine, corticoids or other immunomodulators; (9) pregnancy or breast-feeding; (10) early chemotherapy or radiotherapy before postoperative d 8.

Interventions

Patients were assigned to respective groups by computer-

Table 1 Demographic characteristics of the patients at entry (mean \pm SD)

Group	Group FO (n = 20)	Group SO (n = 21)	t or χ^2	P
Age (yr)	55.80 \pm 10.10	59.19 \pm 10.61	1.047	0.3
Weight (kg)	63.50 \pm 8.86	65.40 \pm 9.20	0.675	0.5
Height (cm)	164.55 \pm 6.68	165.29 \pm 7.60	0.329	0.74
BMI	23.38 \pm 2.38	23.92 \pm 2.84	0.655	0.52
Gender (male/female)	10/10	15/6	1.977	0.16
Diagnosis (colon cancer/rectal cancer)	11/9	12/9	0.019	0.89
TNM stage				
Stage I	0	3	3.2	0.21
Stage II	10	10		
Stage III	10	8		

Table 2 Regimen of daily TPN in the FO and SO groups/kg body weight (g)

Day	Both groups		SO + FO lipids	SO lipids
	Glucose	Nitrogen		
POD+1	3.0	0.18	0.5 SO + 0.1 FO	0.6 SO
POD+2-POD+7	3.0	0.18	1.0 SO + 0.2 FO	1.2 SO

derived block randomization. The pharmacist was the only person who was aware of the randomization list. Both the patients and the investigators were, thus, unaware of the infused drug. Postoperatively, all patients received TPN for consecutive 7 d, as shown in Table 2, through an indwelling central venous catheter or peripheral catheter. Glucose, amino acids, SO emulsion, fat- and water- soluble vitamins as well as trace elements were provided to both groups by infusion pumps for 16-20 h daily in an "All-In-One" manner. In the FO group, the omega-6 lipid content of TPN was partially replaced by omega-3 PUFA (Omegaven, Fresenius-Kabi) up to 0.2 g/kg body weight daily. Thus, in the FO group, the omega-3/omega-6 ratio was 1:3. Calculated on body mass, the nutrition in both groups was isonitrogenous and isocaloric.

Blood samples and analytical methods

For laboratory measurements, 12 mL of whole blood (8 mL serum, 4 mL EDTA) was withdrawn before breakfast in the morning before operation (POD-1) and on the first and eighth days after the operation (POD+1, POD+8). Routine blood test and biochemistry analysis were immediately performed at the Department of Clinical Chemistry, Peking University People's Hospital according to standard procedures. Serum vials for analysis of cytokines such as IL-6 and TNF- α were separated and kept at 2°C-8°C and measured in 24 h. For quantitative detection of IL-6 and TNF- α , enzyme immunoassays were performed according to the manufacturer's instructions with IL-6 or TNF- α enzyme-linked immunosorbent assay (ELISA) kit commercially available from R&D Systems (Minneapolis, MN, USA). Percentage of CD3⁺, CD4⁺, and CD8⁺ lymphocytes was analyzed by flow cytometry (COULTER EPICS ELITE ESP, USA). Fluorescence-labeled antibodies were purchased from BD (Franklin

Table 3 Counts of white blood cells, platelets, and γ -glutamyltranspeptidase before and after operation in the FO group versus the SO group (mean \pm SD)

Group	n	WBC ($\times 10^9$)				Plt ($\times 10^9$)				r-GT (U/L)			
		POD-1	POD+1	POD+8	1	POD-1	POD+1	POD+8	1	POD-1	POD+1	POD+8	1
FO	20	6.76 \pm 2.01	11.20 \pm 2.31	8.17 \pm 1.37	-3.03 \pm 2.46	241.93 \pm 56.62	181.50 \pm 73.47	262.72 \pm 58.63	81.22 \pm 61.58	23.30 \pm 11.55	14.30 \pm 9.26	37.20 \pm 24.49	22.90 \pm 21.35
SO	21	7.03 \pm 2.59	11.70 \pm 3.32	9.03 \pm 2.58	-2.67 \pm 2.58	221.51 \pm 44.20	176.51 \pm 41.25	264.60 \pm 74.13	88.08 \pm 67.07	17.43 \pm 5.80	12.76 \pm 7.89	48.48 \pm 28.41	35.71 \pm 25.34
t		0.381	0.567	1.322	0.447	1.290	0.270	0.090	0.341	2.072	0.574	1.358	1.747
P		0.71	0.57	0.19	0.66	0.20	0.79	0.93	0.74	0.05	0.57	0.18	0.09

1: The margin value for POD+8 minus POD+1.

Table 4 Assessment of inflammatory and immunological parameters before and after operation in the FO group versus the SO group (mean \pm SD)

Group	n	IL-6 (pg/mL)				TNF- α (pg/mL)							
		POD-1	POD+1	POD+8	1	POD-1	POD+1	POD+8	1	POD-1	POD+1	POD+8	1
FO	20	9.02 \pm 23.25	59.66 \pm 31.91	15.23 \pm 8.42	50.64 \pm 32.21	-44.43 \pm 30.53	2.74 \pm 2.00	3.31 \pm 2.85	2.49 \pm 2.06	0.57 \pm 3.46	-0.82 \pm 2.71		
SO	21	10.42 \pm 10.75	42.60 \pm 50.12	34.21 \pm 44.12	32.18 \pm 47.69	-8.39 \pm 69.08	2.48 \pm 3.73	2.66 \pm 2.76	2.94 \pm 3.12	0.18 \pm 4.50	0.27 \pm 1.67		
t		0.249	1.292	1.935	1.445	2.141	0.270	0.738	0.544	0.312	1.570		
P		0.804	0.204	0.066	0.156	0.039	0.789	0.465	0.590	0.757	0.125		

Lakes, NJ, USA) and flow-check fluorospheres were obtained from Beckman-Coulter (Fullerton, CA, USA). Cytokines and percentage of CD3⁺, CD4⁺, and CD8⁺ lymphocytes were analyzed at the Department of Clinical Chemistry, Peking Union Medical College Hospital.

Outcomes of the patients were evaluated considering mortality during the hospital stay, length of postoperative hospital stay, and occurrence of infectious complications.

Statistical analysis

Data were expressed as mean \pm SD and tested for statistical significance using the software SPSS (version 10.0). Analysis of variance or Student's *t*-test or chi-square test was used in statistical analyses. *P* < 0.05 was considered statistically significant.

RESULTS

Clinical characteristics of patients

Only one patient withdrew from the study because of the unresectable disease, whereas 41 patients completed the study, without changes in medication. There were no statistically significant differences between the two groups of patients at entry concerning the clinical characteristics (Table 1). Blood test, biochemistry, serum levels of IL-6 and TNF- α , percentage of CD4⁺, CD8⁺ lymphocytes, and ratios of CD4⁺/CD8⁺ are shown in Tables 3 and 4. Both lipid regimens were well tolerated with no adverse events in terms of bleeding complication.

Clinical outcomes

No death occurred in both groups during the hospital stay, and only one case had incision infection in each group. Therefore, no statistical significant difference was found when stratified to death and occurrence of infectious complications. Although patients in the FO group trended

to need a shorter postoperative hospital stay, no statistically significant difference was found (17.45 ± 4.80 d *vs* 19.62 ± 5.59 d, *P* = 0.19).

Laboratory parameters

Seven days after parenteral nutrition, no statistically significant difference was observed with respect to routine blood test and biochemical evaluation. White blood cell count and serum level of γ -GT in the FO group were lower than those in the SO group (Table 3). Compared with POD+1, serum IL-6 levels on POD+8 were significantly lower in group FO than in reference group (Table 4, -44.43 ± 30.53 *vs* -8.39 ± 69.08 , *P* = 0.039). Simultaneously, the ratios of CD4⁺/CD8⁺ were significantly increased in the FO group (Table 5, 0.92 ± 0.62 *vs* 0.25 ± 1.22 , *P* = 0.035) compared with the reference group. In addition, depression of serum TNF- α levels (-0.82 ± 2.71 *vs* 0.27 ± 1.67 , *P* = 0.125) and elevation of CD3⁺ and CD4⁺ lymphocyte percentage (12.85 ± 11.61 *vs* 3.84 ± 19.62 , *P* = 0.081, 17.80 ± 10.86 *vs* 9.66 ± 17.55 , *P* = 0.084, respectively) were higher in the FO group than in the reference group (Tables 4 and 6).

DISCUSSION

To obtain a homogenous population, we selected patients with colorectal cancer staging TNM I - III to undergo radical resection. Patients were enrolled consecutively and randomly assigned to receive TPN supplemented with either SO or SO + FO emulsion. There were no statistically significant differences between the two groups at entry.

It was reported that reduction in platelet aggregation can be modified by increasing omega-3 long-chain fatty acid content of platelet phospholipids in humans^[18]. In a randomized, controlled double-blind study, Heller AR

Table 5 Assessment of inflammatory and immunological parameters before and after operation in the FO group versus the SO group (mean \pm SD)

Group	n	CD8 ⁺ (%)			Ratio of CD4 ⁺ /CD8 ⁺						
		POD-1	POD+1	POD+8	1	2	POD-1	POD+1	POD+8	1	2
FO	20	25.89 ± 8.09	28.87 ± 7.63	25.46 ± 7.20	2.99 ± 7.20	-3.41 ± 5.79	1.54 ± 0.79	0.89 ± 0.52	1.80 ± 0.74	-0.65 ± 0.69	0.92 ± 0.62
SO	21	26.12 ± 10.95	26.50 ± 12.08	26.31 ± 9.85	0.38 ± 13.07	-0.19 ± 10.12	1.32 ± 0.50	1.27 ± 1.34	1.52 ± 0.69	-0.05 ± 1.28	0.25 ± 1.22
t		0.079	0.747	0.316	0.786	1.244	1.067	1.195	1.269	1.854	2.186
P		0.937	0.460	0.754	0.437	0.221	0.293	0.239	0.212	0.071	0.035

Table 6 Assessment of inflammatory and immunological parameters before and after operation in the FO group versus the SO group (mean \pm SD)

Group	n	CD3 ⁺ (%)					CD4 ⁺ (%)				
		POD-1	POD+1	POD+8	1	2	POD-1	POD+1	POD+8	1	2
FO	20	65.81 ± 9.52	56.43 ± 12.57	69.28 ± 9.42	-9.38 ± 9.88	12.85 ± 11.61	35.69 ± 11.48	23.68 ± 10.69	41.48 ± 9.51	-12.02 ± 10.79	17.80 ± 10.86
SO	21	57.41 ± 11.11	59.73 ± 16.06	63.57 ± 10.26	2.32 ± 20.36	3.84 ± 19.62	30.46 ± 9.74	24.41 ± 15.87	34.07 ± 10.17	-6.05 ± 17.95	9.66 ± 17.55
t		2.591	0.731	1.851	2.320	1.799	1.575	0.173	2.405	1.281	1.775
P		0.013	0.469	0.072	0.026	0.081	0.123	0.864	0.021	0.208	0.084

1: The margin value for POD+1 minus POD-1; 2: The margin value for POD+8 minus POD+1.

and colleagues^[19,20] demonstrated that no coagulation and platelet abnormalities are evoked by fish oil supplementation as high as 0.2 g/kg per day for five postoperative days. In the present study, the change in platelet counts showed no statistical difference between the two groups. Neither bleeding complication nor other adverse events were observed. This is in line with the notion that a short-term parenteral administration of omega-3 fish oil is safe^[9,21]. In addition, our results demonstrate that the serum level of γ -GT on POD+8 in the FO group was lower than that in the SO group. Heller AR *et al*^[20] found that after a major abdominal tumor surgery, fish oil supplementation could improve liver and pancreas function. Animal experiments have demonstrated improved perfusion and fewer translocations of viable bacteria from the gut into the mesenteric lymph nodes and liver after omega-3 fatty acid infusion in rats^[22,23]. Therefore, our results suggest that parenteral nutrition supplemented with omega-3 fish oil might protect liver function after a major abdominal operation in colorectal cancer patients.

Omega-3 and -6 PUFAs are essential for humans and must be nutritionally provided. Recently, omega-3 PUFA deficiency has been recognized and appreciated^[2]. After intravenous administration, EPA and docosahexaenoic acid (DHA) promptly incorporate into the cell membrane, compete with arachidonic acid (AA) in the cyclooxygenase and 5-lipoxygenase pathways, resulting in a reduced generation of diene prostanoids (e.g. PGE₂, PGI₂, TXA₂) and tetraene leukotrienes (e.g. LTB₄), derived from AA in favor of the corresponding triene prostanoids (e.g. PGE₃, PGI₃, TXA₃) and pentaene leukotriene (LTB₅) derived from EPA^[13,24]. In a randomized controlled trial, Köller *et al*^[17] demonstrated that release of 5-series leukotrienes from isolated leukocytes stimulated with Ca-ionophore is increased in patients receiving fish oil. Leukotrienes

have numerous effects on inflammatory and immune functions, such as leucocyte-endothelial interaction, lymphocyte proliferation, and induction of cytokine gene expression (e.g. IL-1, IL-6, or TNF- α)^[25,26]. In a randomized controlled study, Wachtler *et al*^[25] showed that the systemic levels of IL-10, IL-6 and TNF- α are significantly decreased in surgical patients 5 d after administration of TPN enriched with omega-3 fatty acids. In another clinical trial, Weiss *et al*^[27] also found that IL-6 levels are significantly decreased and TNF- α release from monocytes is also decreased in patients receiving fish oil perioperatively. In addition, HLA-DR expression induced by monocytes, an indicator of compensatory potential required to balance immune response, is significantly decreased^[28,29]. Mayer *et al*^[13] displayed that neutrophil function is significantly improved in patients receiving omega-3 fatty acids, including leukotriene generation and respiratory burst. In our study, serum IL-6 levels were significantly lower in the FO group than t in the reference group. This is in agreement with the previous reports^[25,27,30]. Simultaneously, the ratios of CD4⁺/CD8⁺ were significantly increased in the FO group. In addition, depression of serum TNF- α levels and elevation of CD3⁺ and CD4⁺ lymphocyte percentage were noted in the FO group. In an experimental animal model, administration of parental fish oil during sepsis could prevent sepsis-induced suppression of lymphocyte proliferation and IL-2 release^[31]. These findings suggest that supplementation of omega-3 PUFA may restrain inflammatory response, modulate lymphocyte proliferation, and maintain the function of immunocompetent cells under inflammatory conditions such as surgical trauma.

The lower magnitude of postoperative inflammatory response to administration of omega-3 fatty acids may have a favorable impact on clinical outcomes of patients with CRC. A shorter postoperative hospital

stay was noted in our study. No statistically significant difference was found when stratified to death and occurrence of infectious complications. In a cohort of elective postoperative patients, mortality is such a rare event that changes in mortality is underpowered to be detected. Various factors may influence the outcomes of surgical patients. A short single nutritional intervention is unlikely to produce extensive effects on the outcomes of postoperative patients. Recently, in a randomized controlled trial, Weiss *et al*^[27] have demonstrated a shorter postoperative ICU and hospital stay, and a lower rate of severe infections in patients administering omega-3 fish oil perioperatively beginning on POD-1. These results suggest that supplementation with omega-3 fatty acids may have a more favorable effect on the outcomes of CRC patients after a major surgery.

In conclusion, postoperative supplementation of omega-3 fatty acids may have a favorable effect on the outcome of colorectal cancer patients by lowering the magnitude of inflammatory responses and modulating the immune response. Perioperative administration of omega-3 fish oil may have a more favorable effect on the outcome of CRC patients after a major surgery. Further prospective, randomized controlled trials are required to delineate this effect in a larger number of patients.

COMMENTS

Background

Omega-3 and -6 polyunsaturated fatty acids (PUFAs) are essential for humans and must be nutritionally provided. After incorporated into cell membrane, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) promptly compete with arachidonic acid (AA) on the cyclooxygenase and 5-lipoxygenase pathways, resulting in a reduced generation of diene prostanoids (e.g. PGE₂, PGI₂, TXA₂) and tetraene leukotrienes (e.g. LTB₄), derived from AA and are in favor of triene prostanoids (e.g. PGE₃, PGI₃, TXA₃) and pentaene leukotriene (LTB₅) derived from EPA. Therefore, the benefit of omega-3 fatty acids is to suppress the system inflammatory response and decrease the risk of postoperative thrombosis, which has been appreciated recently.

Research frontiers

Recently, clinical nutrition has attempted to combine caloric support with modulation of the immune response. Several new generations of lipid emulsion containing n-3 lipids have been introduced, and immunonutrition has become the hot spot or an important area in this research field.

Innovations and breakthroughs

Although several studies have demonstrated the beneficial effects of omega-3 fatty acid supplementation on the outcome or immune competence of patients, randomized controlled clinical trials focusing on the use of parenteral fish oil are scarce, especially in colorectal cancer patients. To obtain a homogenous population, patients with colorectal cancer staging TNM I-III were selected to undergo radical resection, and concomitant disorders were restricted according to the exclusion criteria. Depression of inflammatory parameters, such as serum level of IL-6 and TNF- α was observed. Elevation of CD4⁺/CD8⁺ ratio, CD3⁺ and CD4⁺ lymphocyte percentage, was noted after administration of omega-3 fatty acids supplementation. Meanwhile, a near-significant improvement in clinical outcome was demonstrated. Patients accepted omega-3 fatty acid supplementation trended to need a shorter postoperative hospital stay. In view of the authors, postoperative supplementation of omega-3 fatty acids may have a favorable effect on the outcome of colorectal cancer patients undergoing radical resection by lowering the magnitude of inflammatory responses and modulating the immune response.

Applications

By summing up the available data from surgical patients, we conclude that fish oil

should be included in parenteral nutrition yielding positive impact on the outcome of patients.

Terminology

Immunonutrition, which combines caloric support and modulation of the immune response, has become the hot spot in this research field.

Peer review

This article on the effect of omega-3 fatty acid supplementation on colorectal cancer is interesting.

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RAPID COMMUNICATION

Analysis of risk factors for the interval time, number and pattern of hepatic metastases from gastric cancer after radical gastrectomy

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Abstract

AIM: To analyze the risk factors for interval time, number and pattern of hepatic metastases from gastric cancer after radical gastrectomy, and provide evidence for predicting and preventing hepatic metastasis from gastric cancer after radical gastrectomy.

METHODS: A retrospective study of 87 patients with hepatic metastasis who underwent radical gastrectomy for gastric cancer from 1996 to 2001. The data was analyzed to evaluate significant risk factors for interval time, number and pattern of hepatic metastases originating from gastric cancer after radical gastrectomy.

RESULTS: The size of gastric cancer and lymph node metastases were independently correlated with the interval time of hepatic metastases; the depth of invasion was independently correlated with the number of hepatic metastases; while the depth of invasion and Lauren classification were independently correlated with the pattern of hepatic metastases.

CONCLUSION: We evaluated the interval time of hepatic metastases with the size of gastric cancer and lymph node metastases. The depth of invasion could be used to evaluate the number of hepatic metastases, while the depth of invasion and the Lauren classification could be used to evaluate the pattern of hepatic metastases in patients who underwent radical gastrectomy.

INTRODUCTION

Over 60% patients with gastric cancer are diagnosed at an advanced stage in China, and the overall 5-year survival remains less than 50%. Advances in the operative techniques and perioperative care have reduced the operative mortality and morbidity, but have not improved the stage-specific cancer survival rate. Long-term survival after radical gastrectomy for gastric cancer in China is very poor^[1,2]. A number of prospective trials have failed to show a survival advantage with more extensive gastric resection^[3-5] and extensive lymphadenectomy^[6,7]. Moreover, patients with advanced stage disease continue to have disease recurrence at a high rate, and the recurrence is mainly focused in specific areas (locoregional, peritoneal, or liver). Hepatic metastasis from gastrointestinal carcinoma is a frequent and critical problem. Several studies have shown that hepatic resection for metastatic tumors from colorectal cancer is associated with improved outcome^[8-10], and as this procedure has become safer, the indications for its use in such situations have expanded. However, in the case of liver metastases from gastric carcinoma, which is equally common, very few patients are candidates for hepatic resection because of the presence of multiple, widespread, bilobar metastases. As a result, spread of disease to different sites such as peritoneal dissemination, lymph node metastases, and distant metastasis is very common. There are very few reports on surgical resection of hepatic metastases from gastric cancer, and the results are disappointing^[11,12]. It is important to determine the

risk factors for hepatic metastasis in order to improve the survival rate of gastric cancer after radical gastrectomy. The aims of this study were to identify independent risk factors for interval time, number and pattern of hepatic metastases originating from gastric cancer after radical gastrectomy, and to propose steps for the prevention of hepatic metastases after radical gastrectomy.

MATERIALS AND METHODS

Patients

Between 1996 and 2001, 87 patients with gastric carcinoma who underwent radical gastrectomy in the Gastrointestinal Cancer Department, Tianjin Cancer Hospital, Tianjin Medical University, Tianjin were selected. These patients consisted of 32 with the primary tumor located in the proximal stomach, 8 with tumor in middle stomach and 45 with distal stomach tumor. There were 77 men and 10 women, with a mean age of 62 years (range 38-78). All patients had complete resection of the primary gastric cancer. No patient died during the initial hospital stay or for 1 mo after surgery. Follow-up ranged from 3 mo to 60 mo (median 32 mo).

Methods

The demographic and clinical information comprised of the following parameters: age, gender, interval time between radical gastrectomy and hepatic metastases, surgical procedure and postoperative chemotherapy.

Macroscopically, the size of the primary tumor, primary tumor location, number and pattern of metastasis, and metastatic interval time were recorded. Microscopic features evaluated were histologic differentiation, depth of invasion of the primary tumor, lymph node metastasis, Lauren classification, presence of vascular invasion, presence of neural invasion, and peritoneal metastasis. In patients with multiple hepatic metastases, the pathologic findings of the largest tumor were taken as representative of the other tumors, because all the tumors showed similar pathologic features. The histologic types of the primary gastric cancer and the hepatic metastasis were determined according to the World Health Organization Classification^[6]. The depth of invasion, extent of lymph node metastasis, Lauren classification, and presence of vascular and neural invasion of the primary gastric cancer were also evaluated. The pathologic diagnosis and classification of the primary cancer were performed by at least two pathologists using the TNM classification of the UICC^[7], and results of gastric cancer study in surgery and pathology in Japan^[8].

Statistical analysis

The various clinicopathologic factors were analyzed by the method of Kaplan and Meier, and Log-rank test was used to determine univariate significance. Factors that were deemed of potential importance on univariate analysis ($P < 0.05$) were included in the multivariate analysis. Logistic regression or Cox proportional hazards model were used for multivariate analysis. Significance was defined as $P < 0.05$. Surgical procedures were classified as proximal radical gastrectomy, esophago-gastric resection, distal radical

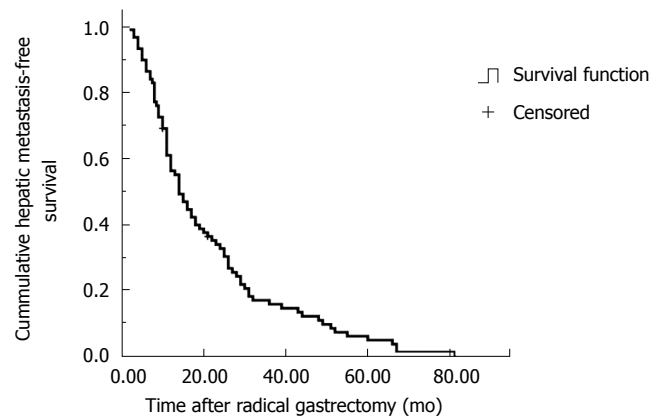


Figure 1 Kaplan-Meier cumulative hepatic metastasis-free survival plot for the entire cohort of 87 patients who underwent gastrectomy of gastric adenocarcinoma.

gastrectomy or total gastrectomy. Statistical analysis was performed using the statistical analysis program package (SPSS 13.0, Chicago, IL).

RESULTS

Clinicopathologic data

Histological analysis revealed that all the primary gastric tumors were adenocarcinomas. There was no evidence of metastases to other organs or peritoneal dissemination determined by imaging studies (such as B ultrasonography, CT or MRI), before curative gastrectomy was performed. Esophago-gastric resection was performed on 23 patients, proximal radical gastrectomy 8 patients, distal radical gastrectomy 45 patients and total gastrectomy 11 patients. The median interval time between gastrectomy and hepatic metastasis in all patients was 14 mo (range 3-31).

Patient outcome

The time interval for hepatic metastasis free survival for the 87 patients who underwent radical gastrectomy is shown in Figure 1. Factors associated with interval time of hepatic metastases after radical gastrectomy are shown in Table 1. The actuarial interval time for < 6 mo, 6-12 mo, 12-36 mo, and more than 36 mo for hepatic metastases after primary radical gastrectomy were 9 (10.3%), 26 (29.9%), 38 (43.7%) and 14 (16.1%) respectively. The median hepatic metastasis-free survival time was 14.0 mo. With univariate analysis, five factors were found to have statistically significant association with the interval time of hepatic metastases after radical gastrectomy: size of gastric cancer, lymph node metastasis, No. 12 lymph node group metastases, No. 8 lymph node group metastases, and Lauren classification. Only the size of gastric cancer and lymph node metastasis showed significant correlation with the interval time of hepatic metastases using the Cox proportional hazards model analysis.

Factors associated with the number of hepatic metastases after radical gastrectomy are shown in Table 2. The number of patients with solitary metastasis and multiple metastases after primary radical gastrectomy were 11 (12.6%), and 76 (87.4%) respectively. With univariate analysis, three factors were found to have statistically significant association

Table 1 Univariate and multivariate analysis of clinicopathologic factors potentially associated with the interval time of hepatic metastases after radical gastrectomy

Factor	n	Cases of hepatic metastases after surgery				Univariate <i>P</i> value	Multivariate <i>P</i> value	Odds ratio
		< 6 mo	6-12 mo	12-36 mo	> 36 mo			
Age								
≤ 50 yr	13	0	5	7	1	0.634	0.334	0.800
51- 69 yr	55	6	16	22	11	-	-	-
≥ 70 yr	19	3	5	9	2	-	-	-
Gender								
Male	77	9	24	31	13	0.309	0.149	1.949
Female	10	0	2	7	1	-	-	-
Gastric carcinoma								
Size								
≤ 5 cm	39	2	5	21	11	0.001	0.019	1.989
> 5 cm	48	7	21	17	3	-	-	-
Location								
Proximal	25	2	10	9	4	0.512	0.966	1.007
Middle	17	1	6	8	2	-	-	-
Distal	43	5	9	21	8	-	-	-
Diffuse	2	1	1	0	0	-	-	-
Histologic differentiation								
Poorly undifferentiated	42	6	14	17	5	0.454	0.091	0.629
Well, moderately	45	3	12	21	9	-	-	-
Vascular invasion								
Absent	70	6	19	32	13	0.296	0.134	1.928
Present	17	3	7	6	1	-	-	-
Neural invasion								
Absent	74	7	20	33	14	0.232	0.383	1.502
Present	13	2	6	5	0	-	-	-
Depth of invasion ¹								
T1	4	0	1	2	1	0.085	0.400	0.845
T2	26	1	5	14	6	-	-	-
T3	48	5	20	18	5	-	-	-
T4	9	3	0	4	2	-	-	-
Lymph node metastases ¹								
N1	31	0	3	19	9	< 0.001	0.001	1.892
N2	37	3	17	13	4	-	-	-
N3	19	6	6	6	1	-	-	-
No. 12 lymph node group metastases								
Absent	75	5	21	35	14	0.011	0.880	0.915
Present	12	4	5	3	0	-	-	-
No. 8 lymph node group metastases								
Absent	72	5	20	33	14	0.034	0.647	0.792
Present	15	4	6	5	0	-	-	-
Lauren classification								
Intestinal	36	4	8	15	9	0.037	0.331	1.171
Diffuse	29	5	13	10	1	-	-	-
Mixed	22	0	5	13	4	-	-	-
Surgical procedure								
Esophago-proximal	23	2	1	17	3	0.056	0.481	1.098
Proximal subtotal	8	1	4	2	1	-	-	-
Distal subtotal	45	4	18	14	9	-	-	-
Total	11	2	3	5	1	-	-	-
Ascites								
Absent	57	8	17	22	10	0.338	0.608	0.866
Present	30	1	9	16	4	-	-	-
Soft tissue invasion								
Absent	47	6	18	17	6	0.170	0.136	0.559
Present	40	3	8	21	8	-	-	-

¹According to pTNM classification of UICC; Esophago-proximal, resection of the distal esophagus and proximal stomach; total, resection of the whole stomach.

with the number of hepatic metastases after radical gastrectomy: size of gastric cancer, depth of invasion, and Lauren classification. Only the depth of invasion of primary gastric tumor showed significant correlation with the number of hepatic metastasis, using the Logistic regression multivariate analysis.

Factors associated with the pattern of hepatic metastases after radical gastrectomy are depicted in Table 3. The pattern of hepatic metastases comprised of three subtypes (H1-3, according to the general rules for gastric cancer study in surgery and pathology in Japan). H1 subtype indicates that all the hepatic metastatic lesions are unilobar in

Table 2 Univariate and multivariate analysis of clinicopathologic factors potentially associated with the number of hepatic metastases after radical gastrectomy

Factor	n	Cases of hepatic metastases after surgery		Univariate <i>P</i> value	Multivariate <i>P</i> value	Odds ratio
		Solitary	Multiple			
Age						
≤ 50 yr	13	2	11	0.171	0.398	2.480
51-69 yr	55	9	46	-	-	-
≥ 70 yr	19	0	19	-	-	-
Gender						
Male	77	10	67	0.798	0.732	0.392
Female	10	1	9	-	-	-
Gastric carcinoma						
Size						
≤ 5 cm	39	10	29	0.001	0.133	12.271
> 5 cm	48	1	47	-	-	-
Location						
Proximal	25	3	22	0.678	0.539	1.487
Middle	17	1	16	-	-	-
Distal	43	7	36	-	-	-
Diffuse	2	0	2	-	-	-
Histologic differentiation						
Poorly undifferentiated	42	4	38	0.398	0.797	1.359
Well, moderately	45	7	38	-	-	-
Vascular invasion						
Absent	70	11	59	0.08	0.998	-
Present	17	0	17	-	-	-
Neural invasion						
Absent	74	11	63	0.137	0.998	-
Present	13	0	13	-	-	-
Depth of invasion ¹						
T1	4	3	1	< 0.001	0.046	8.799
T2	26	6	20	-	-	-
T3	48	2	46	-	-	-
T4	9	0	9	-	-	-
Lymph node metastases ¹						
N1	31	7	24	0.060	0.991	0.989
N2	37	4	33	-	-	-
N3	19	0	19	-	-	-
No. 12 lymph node group metastases						
Absent	75	11	64	0.156	0.998	-
Present	12	0	12	-	-	-
No. 8 lymph node group metastases						
Absent	72	10	62	0.444	0.428	0.125
Present	15	1	14	-	-	-
Lauren classification						
Intestinal	36	9	27	0.009	0.288	3.261
Diffuse	29	0	29	-	-	-
Mixed	22	2	20	-	-	-
Surgical procedure						
Esophago-proximal	23	3	20	0.056	0.395	1.953
Proximal subtotal	8	1	7	-	-	-
Distal subtotal	45	7	38	-	-	-
Total	11	0	11	-	-	-
Ascites						
Absent	57	8	49	0.338	0.718	0.544
Present	30	3	27	-	-	-
Soft tissue invasion						
Absent	47	5	42	0.170	0.950	1.102
Present	40	6	34	-	-	-

¹According to pTNM classification of UICC; Esophago-proximal, resection of the distal esophagus and proximal stomach; total, resection of the whole stomach.

distribution. H2 subtype suggests metastases in both lobes. H3 subtype refers to scattered metastases in both lobes. The number of patients with H1, H2 and H3 metastases after primary radical gastrectomy were 12 (13.8%), 31 (35.6%) and 44 (50.6%) respectively. With univariate analysis, four factors were found to have statistically significant association

with the number of hepatic metastasis after radical gastrectomy: size of gastric cancer, depth of invasion, Lauren classification, and vascular invasion. Only the depth of invasion and Lauren classification showed significant correlation with the pattern of hepatic metastasis, based on Logistic regression multivariate analysis.

Table 3 Univariate and multivariate analysis of clinicopathologic factors potentially associated with the pattern of hepatic metastases after radical gastrectomy

Factor	n	Cases of hepatic metastases after surgery			Univariate <i>P</i> value	Multivariate <i>P</i> value	Odds ratio
		H1	H2	H3			
Age							
≤ 50 yr	13	2	7	4	0.221	0.098	2.034
51-69 yr	55	9	15	31	-	-	-
≥ 70 yr	19	1	9	9	-	-	-
Gender							
Male	77	11	30	36	0.126	0.168	0.202
Female	10	1	1	8	-	-	-
Gastric carcinoma							
Size							
≤ 5 cm	39	10	13	16	0.014	0.618	0.758
> 5 cm	48	2	18	28	-	-	-
Location							
Proximal	25	3	5	17	0.180	0.985	1.006
Middle	17	1	8	8	-	-	-
Distal	43	8	18	17	-	-	-
Diffuse	2	0	0	2	-	-	-
Histologic differentiation							
Poorly undifferentiated	42	4	12	26	0.118	0.621	0.763
Well, moderately	45	8	19	18	-	-	-
Vascular invasion							
Absent	70	12	28	30	0.011	0.190	4.267
Present	17	0	3	14	-	-	-
Neural invasion							
Absent	74	12	28	34	0.087	0.844	1.235
Present	13	0	3	10	-	-	-
Depth of invasion ¹							
T1	4	3	1	0	< 0.001	0.037	2.078
T2	26	6	13	7	-	-	-
T3	48	3	13	32	-	-	-
T4	9	0	4	5	-	-	-
Lymph node metastases ¹							
N1	31	7	13	11	0.073	0.194	1.634
N2	37	5	13	19	-	-	-
N3	19	0	5	14	-	-	-
No. 12 lymph node group metastases							
Absent	75	12	25	38	0.256	0.224	0.277
Present	12	0	6	6	-	-	-
No. 8 lymph node group metastases							
Absent	72	11	26	35	0.603	0.677	1.483
Present	15	1	5	9	-	-	-
Lauren classification							
Intestinal	36	10	17	9	0.001	0.005	2.552
Diffuse	29	0	8	21	-	-	-
Mixed	22	2	6	14	-	-	-
Surgical procedure							
Esophago-proximal	23	3	8	12	0.807	0.698	1.122
Proximal subtotal	8	1	2	5	-	-	-
Distal subtotal	45	8	16	21	-	-	-
Total	11	0	5	6	-	-	-
Ascites							
Absent	57	9	21	27	0.644	0.984	1.012
Present	30	3	10	17	-	-	-
Soft Tissue Invasion							
Absent	47	6	22	19	0.057	0.765	1.179
Present	40	6	9	25	-	-	-

¹According to pTNM classification of UICC; Esophago-proximal, resection of the distal esophagus and proximal stomach; total, resection of the whole stomach.

DISCUSSION

The outcome of gastric cancer has not shown a significant improvement with the current treatment approaches. Although early detection improves the prognosis, most patients with gastric cancer are identified at an advanced

stage and have a poor prognoses despite developments in surgical techniques and the use of anticancer chemotherapy. In addition, patients with advanced cancer have very high rate of tumor recurrence, which is nearly always lethal^[1,13]. Hepatic metastasis is the most frequent presentation of recurrent gastric cancer after radical

gastrectomy. The prognosis of gastric cancer in patients with hepatic metastasis is poor, and the best method of treatment remains unclear. The benefit of resection of hepatic metastases from gastric carcinoma is not widely accepted, and nonsurgical treatments, including the use of systemic or hepatic artery infusion chemotherapy has not produced satisfactory results. Hepatic resection of metastatic tumors from colorectal cancer is considered the standard of care, however, patients with metastatic liver tumors from gastric cancer are rarely considered good candidates for surgical treatment because most cases have multiple metastases and peritoneal dissemination^[12,14]. Only 10% to 20% of patients with hepatic metastases from gastric cancer after gastrectomy are suitable for surgical treatment; the procedure has a median survival of 5-8 mo, with 15%-50% survival at 1 year and the 5-year survival rate is close to zero^[15-19].

The prolonged disease-free interval after gastrectomy in long-term survivors suggests that these tumors have a more indolent biologic character. Imamura *et al* reported that the prognosis of patients with an interval time > 1 year was better than that of patients whose interval time from gastrectomy to hepatic metastasis was < 1 year^[19]. This feature may be useful in designing an adjuvant treatment program for such patients after surgical resection. Ambiru *et al* observed that the interval time from gastrectomy to hepatic metastases was an independent factor in determining the prognoses of patients who underwent hepatic resection^[20]. Our findings show that the size of gastric cancer (OR = 1.989, $P = 0.019$) and lymph node metastases (OR = 1.892, $P = 0.001$) has significant correlation with the interval time of hepatic metastases, based on the results of the Cox proportional hazards model analysis. Lee *et al* proposed that both size and pattern of lymph node metastases provide prognostic information on the survival rate of gastric cancer patients^[21]. Dong *et al* observed that the regional lymph node metastatic rate of patients with a mean gastric tumor size > 3 cm was greater than that of patients with a mean gastric tumor size less than 2 cm ($P < 0.01$)^[22]. Baba *et al* reported absence of metastasis in lesions less than 1 cm in diameter, and the incidence of positive nodes increased with increasing size of the primary gastric tumor^[23]. These observations and our own findings indicate that the size of the primary gastric tumor is associated with lymph node metastases, which is the most important factor in determining the recurrence of gastric cancer after radical gastrectomy, and has critical impact on the interval time from gastrectomy to hepatic metastases.

The presence of solitary or metachronous hepatic metastases are significant determinants for a favorable prognosis after radical gastrectomy. In liver metastases from colorectal carcinoma, the number of metastases is no longer considered an important predictor of long-term survival, if complete excision is achieved, survival after resection of up to eight metastases is similar to that after resection of a solitary metastasis^[24]. The difference in the results between colorectal and gastric metastases is believed to reflect the aggressive biologic behavior of gastric cancer. Indications for resection

of hepatic metastases should be based on the biologic character of the primary tumor. In our study, all patients who underwent radical gastrectomy and resection of the hepatic metastases had metachronous metastases. Solitary hepatic metastasis was seen in only 11 cases, the remaining 76 patients had multiple hepatic metastases. In the present study, we attempted to correlate several clinical and histological factors with the number of hepatic metastases from gastric cancer after radical gastrectomy. By univariate analysis, a number of variables that affected the outcome were identified. However, by multivariate logistic regression analysis, only the depth of invasion of the primary gastric tumor was an independent risk factor for hepatic metastasis (OR = 8.799; $P = 0.046$; 95% CI, 0.789-79.280). The depth of invasion of the primary gastric tumor and the number of metastatic lymph nodes were considered the most reliable prognostic indicators, with the strongest influence on the risk of recurrence after radical gastrectomy^[25-30]. Michael *et al* reported that the depth of primary gastric tumor was associated with higher rates of metastasis to the peritoneum (locoregional, peritoneal, or distant), and was associated with a significantly shorter median time from recurrence to death^[31]. Patients who had surgery for early gastric cancer had an excellent chance of long-term survival, whereas patients with serosal involvement had a very poor prognosis^[32-34]. These studies suggested that greater depth of primary gastric cancer was associated with higher propensity to develop hematogenous metastases.

Usually, patients with hepatic metastasis were initially found to have multiple lesions in the liver. According to the general rules for the study of gastric cancer in surgery and pathology in Japan, hepatic metastases from gastric cancer should be divided into three subtypes (H1-3). H1 subtype refers to unilobar distribution metastasis. H2 subtype indicates metastatic lesions in both lobes. H3 subtype of hepatic metastases indicates the presence of numerous metastatic lesions in both lobes. Irrespective of whether the number of hepatic lesions is single or multiple, H1 subtype of hepatic metastases is an absolute indication for hepatic resection. However, most experts consider H2 and H3 subtypes as contraindications for hepatic resection^[12,17,19,20,35]. Chen *et al* reported that the median survival time of H1 subtype treated with hepatic resection was longer than that of H2 or H3 subtypes treated without surgery ($P = 0.0072$)^[36]. In the present study, the number of patients with H1, H2, and H3 metastases were 12, 31 and 44 respectively. Only the depth of invasion of the primary gastric cancer and Lauren classification showed significant correlation with the number of hepatic metastases, based on Logistic regression multivariate analysis. The Lauren classification divides tumors into intestinal type, diffuse type, and mixed (unclassifiable) type^[37]. The Lauren diffuse type is associated with significantly worse prognosis for gastric cancer compared to the other types^[38-40]. The depth of primary gastric tumor was associated with higher rate of recurrence of gastric cancer after gastrectomy^[31]. These observations suggest that both the Lauren diffuse type and advanced depth of primary tumor are important

risk factors for hepatic metastasis from gastric cancer after radical gastrectomy.

The following conclusions can be drawn based on the present study. The size of the primary tumor (> 5 cm) and advanced lymph node metastases are important predictors of the interval time for hepatic metastases from gastric cancer after radical surgery. The prognostic value of the depth of the primary gastric cancer is independent of the number of hepatic metastases from gastric cancer after radical surgery. Both advanced depth of the primary gastric cancer and diffuse Lauren type are associated with H2 and H3 subtypes of hepatic metastases from gastric cancer after radical surgery.

COMMENTS

Background

Several studies have shown that resection is the ideal treatment for hepatic metastases from gastric cancer after radical gastrectomy. Assessment of the number and the pattern of hepatic metastases are very important for the surgeon to ascertain suitable candidates for surgical treatment. In addition, the interval time of hepatic metastases can determine in part the biologic character of the tumor. However, factors which affect the interval time, and the number and pattern of hepatic metastases from gastric cancer after radical surgery remain unclear.

Research frontiers

We observed that the size of the gastric cancer and lymph node metastases are independent risk factors in predicting the interval time of hepatic metastases after radical gastrectomy. In the present study, only the depth of invasion was an independent risk factor for the number of hepatic metastases after radical gastrectomy. In addition, both the depth of invasion and the Lauren classification were found to be independently correlated with the pattern of hepatic metastases.

Innovations and breakthroughs

From the results of this study, we can draw the following conclusions about hepatic metastases from gastric cancer after radical gastrectomy: the size of primary tumor and lymph node metastases are important predictors of the interval time; the depth of the primary gastric cancer is independent of the number of hepatic metastases; both the depth of primary gastric cancer and the Lauren type are associated with the pattern of hepatic metastases.

Applications

The clinicopathological risk factors for hepatic metastases from gastric cancer after radical gastrectomy have been elucidated in the present study. Based on our findings, we can predict hepatic metastases in patients with gastric cancer after radical gastrectomy.

Peer review

The author retrospectively analyzed 87 patients with hepatic metastasis who underwent radical gastrectomy for gastric cancer, and elucidated the risk factors for interval time, number, and pattern of hepatic metastases after curative gastrectomy. The results of this study provide important clues to predicting hepatic metastases in gastric cancer patients after radical gastrectomy.

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CASE REPORT

Ulcerative colitis presenting as leukocytoclastic vasculitis of skin

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Abstract

A number of cutaneous changes are known to occur in the course of inflammatory bowel diseases (IBD), including pyoderma gangrenosum, erythema nodosum, perianal disease, erythematous eruptions, urticaria, and purpura. However, occurrence of skin manifestations prior to the development of ulcerative colitis is a rare occasion. Here, we report a case of ulcerative colitis associated with leukocytoclastic vasculitis in which the intestinal symptoms became overt 8 mo after the development of skin lesions.

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Key words: Leukocytoclastic vasculitis; Ulcerative colitis; Skin; Extra-intestinal; Primary sclerosing cholangitis

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INTRODUCTION

Leukocytoclastic vasculitis is characterized by neutrophilic invasion and fibrinoid necrosis along with endothelial

enlargement in postcapillary venules^[1]. It is a syndrome in which patients most commonly present with palpable purpura on lower extremities and ankles.

Inflammatory bowel disease (IBD) can be associated with skin manifestations^[2]. Association between leukocytoclastic vasculitis and ulcerative colitis is uncommon and in most cases cutaneous leukocytoclastic vasculitis proceeds the intestinal symptoms^[3]. Here, we present a case diagnosed with leukocytoclastic vasculitis 8 mo before the development of ulcerative colitis.

CASE REPORT

A 20-year-old female had a history of itching in arms and legs which began in February 2005. She had no fever, diarrhea, weight loss, hematuria, vision problems, or headache. Anti-allergic medications did not show any favorable effects on the symptoms. Meanwhile, multiple reddish-brown macular lesions developed on her heels spreading upwards to legs and buttocks. She was treated with oral prednisolone at a dose of 20 mg/d. Although old lesions seemed to subside following treatment, new lesions appeared on the back, neck, and arms. Light brown macular lesions united to form plaque-like non-blanching lesions with erythema at the center. Skin biopsy was consistent with leukocytoclastic vasculitis. Figure 1A and B demonstrate the histologic characteristics of the skin biopsy specimen. Although, the dose of prednisolone was increased to 40 mg/d, she did not respond to it, thus, it was discontinued.

She was admitted to our clinic with the complaint of bloody diarrhea in September 2005, eight months after the first appearance of skin lesions. She reported that she passed bloody stool containing mucus, 8-10 times a day. Apart from the scars of old lesions and new light-brown macular lesions around the ankles, physical examination yielded normal results. Results of the laboratory investigations included the followings: 9.870/mm³ white blood cells, 10.6 g/dL hemoglobin, 72 fL MCV, 409.800/mm³ platelets. Peripheral blood smear showed hypochromia, microcytosis and anisocytosis, 29 mm/h of erythrocyte sedimentation rate, 34 C-reactive proteins, 75 U/L ALT (normal: 0-50 U/L), 64 U/L AST (normal: 0-40 U/L), 159 U/L ALP (normal: 40-150 U/L), 272 U/L GGT (normal: 5-64 U/L), and 0.81 mg/dL total bilirubin (normal: 0.2-1.2 mg/dL). Urine analysis was normal. Blood fasting glucose, urea, creatinine, total protein, albumin levels and electrolyte (albumin, sodium, potassium, chloride, and calcium) concentration were within

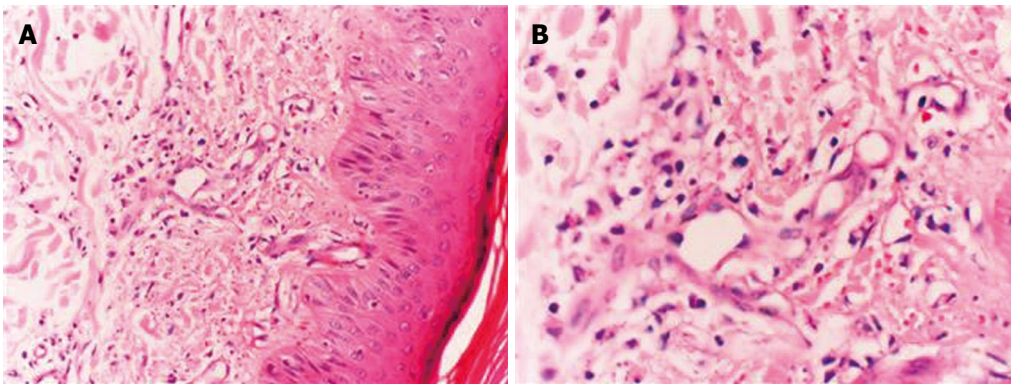


Figure 1 Skin biopsy specimen (HE, $\times 200$) showing polymorphonuclear cells and lymphocyte infiltration in and around the vessels of dermis beneath the multilayered keratinized squamous epithelium with some nuclear debris in the interstitium (A) and fibrin deposits in the vessel wall and nuclear debris (B).



Figure 2 Magnetic resonance cholangiopancreatography demonstrating ductal irregularities and beading appearance in the distal branches of the right and left hepatic canals.

normal limits. HBsAg (-), anti-HCV (-), anti-HIV (-), thyroid function tests, anti-TPO antibody, and anti-TG antibody were normal. Other selected laboratory tests showed serum folic acid of 4.82 ng/mL (normal > 3.00 ng/mL), vitamin B₁₂ of 226 pg/mL (normal: 160-980 pg/mL), ferritin of 3.3 ng/mL (normal: 5-148 ng/mL), serum iron of 6 g/dL (normal: 40-170 g/dL), and serum iron binding capacity of 470 g/dL (normal: 250-425 g/dL).

Abdominal ultrasonography revealed a mild edematous appearance in intestinal walls with no other abnormalities. Colonoscopic examination of the terminal ileum revealed a normal mucosa and lumen. The ileocecal valve appeared to be normal. However, mucosa of the entire colon was diffusely hyperemic and edematous with disappearance of the submucosal vascular network, and scattered shallow ulcerations. Histopathological examination of colonic specimen showed chronic mucosal inflammation with cryptic abscesses and distortion, suggestive of ulcerative colitis. Treatment with mesalazine (5-ASA) at a dose of 2 g/d was commenced and the patient was asked to visit one month later.

One month later, her bloody diarrhea and skin lesions disappeared and she passed formed stools once a day. However, liver function tests remained elevated (67 U/L AST, 92 U/L ALT, 165 U/L ALP, and 224 U/L GGT). Thus, quantitative serum immunoglobulin tests were as follows: ANA (-), anti-ds DNA (-), AMA (-), ASMA (-), anti LKM-1 (-), SLA/LP M2 (-), p-ANCA (+), 1.33 g/L C₃ (normal: 0.9-1.8 g/L), 0.15 g/L C₄ (normal: 0.1-0.4 g/L),

19.10 g/L IgG (normal: 7-16 g/L), 1.56 g/L IgM (normal: 0.4-2.3 g/L), and 3.01 g/L IgA (normal: 0.7-4 g/L).

Since serum ALP and GGT values were high and p-ANCA was positive, a magnetic resonance cholangiopancreatography (MRCP) was performed with the suspicion of primary sclerosing cholangitis (PSC). MRCP revealed ductal irregularities at distal branches of the right and left hepatic canals (Figure 2). Thus, ursodeoxycholic acid (UDCA) was started at a dose of 20 mg/kg, once a day, with a presumptive diagnosis of PSC. Following the treatment, her liver function tests returned to normal within two months. She was still well with oral 5-ASA and UDCA treatment at the time when we wrote this paper.

DISCUSSION

Various skin findings can accompany inflammatory bowel disorders^[2]. Skin manifestations occur in about 15% of patients with inflammatory bowel disorders^[4]. Most frequently accompanying skin manifestations are pyoderma gangrenosum and erythema nodosum, while necrotizing vasculitis, cutaneous polyarteritis nodosa and granulomatous perivascularitis are less frequently seen^[5-8]. Although the etiopathogenesis of extra-intestinal manifestations is not clear, a partial defect of immunity common to the skin and intestines has been suggested^[5].

Leukocytoclastic vasculitis is a disorder characterized by neutrophilic infiltration and nuclear debris in postcapillary venules^[1,9]. It is believed to be an immune-complex disorder triggered by various drugs, infections, malignancies, and systemic and autoimmune disorders^[10-13]. Although it usually involves the skin, systemic manifestations such as fever, arthralgia, myalgia or asthenia may also develop^[2]. Leukocytoclastic vasculitis is less frequently seen in patients with ulcerative colitis as compared to other skin manifestations^[14-18]. Clinically, it is generally synchronous with ulcerative colitis. Three previous ulcerative colitis cases who presented leukocytoclastic vasculitis before onset of the intestinal disease have been reported^[3,15]. As reported by Newton *et al*^[15], vasculitic symptoms appear 1 to 6 mo before the onset of intestinal symptoms. Iannone *et al*^[3] reported another case whose intestinal disease occurred 2 years after appearance of vasculitic skin lesions. In our case, leukocytoclastic vasculitis developed 8 mo before the appearance of intestinal symptoms.

One possible explanation of the association between these two disorders can be that the pathogenesis of

both is based on immune mechanisms and deposition of immune complexes in the vascular wall and intestinal mucosa for leukocytoclastic vasculitis and ulcerative colitis, respectively^[19]. Another possible explanation might be that IBD is a systemic disorder involving different tissues (skin, joints, intestine) at different episodes^[3].

Skin lesions of leukocytoclastic vasculitis can be treated with corticosteroids, dapsone, colchicine, or immunosuppressive agents^[1]. The treatment can be directed to the underlying cause (i.e., drugs, infections, malignancies, autoimmune diseases), if it is present. As previously mentioned, cases of leukocytoclastic vasculitis accompanying ulcerative colitis can be effectively treated with colchicine and sulfasalazine. In our case, both intestinal symptoms and skin lesions were successfully treated with 5-ASA.

The other diagnosis of our patient was PSC. It was reported that the prevalence of PSC in patients with ulcerative colitis is 5.5%^[20]. ANCA positivity in patients with PSC is 56%-88%. Patients with PSC can be asymptomatic (25%-45%) at the time of diagnosis^[21]. In our case, it is possible that PSC developed synchronously with leukocytoclastic vasculitis. Not any case in the literature presented PSC along with leukocytoclastic vasculitis.

In conclusion, IBD should be kept in mind as a cause of leukocytoclastic vasculitis, although it is a rare occasion. A careful follow-up of such cases may improve both vasculitis and IBD.

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Association of primary biliary cirrhosis with idiopathic thrombocytopenic purpura

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Abstract

Although both primary biliary cirrhosis (PBC) and idiopathic thrombocytopenic purpura (ITP) are autoimmune diseases, the association of the 2 diseases is rare. Here, we report a case of ITP that developed during the follow-up of PBC in a 74-year-old man. The patient had been diagnosed with PBC 12 years previously, and had received treatment with ursodeoxycholic acid. The platelet count decreased from approximately $60 \times 10^9/L$ to $8 \times 10^9/L$, and the association of decompensated liver cirrhosis (PBC) with ITP was diagnosed. Steroid and immune gamma globulin therapy were successful in increasing the platelet count. Interestingly, human leukocyte antigen genotyping detected the alleles DQB1*0601 and DRB1*0803, which are related to both PBC and ITP in Japanese patients. This case suggests common immunogenetic factors might be involved in the development of PBC and ITP.

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Key words: Primary biliary cirrhosis; Idiopathic thrombocytopenic purpura; Anti-platelet autoantibody; Platelet surface glycoprotein complex; Human leukocyte antigen

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INTRODUCTION

Primary biliary cirrhosis (PBC) is a chronic liver disease characterized by progressive biliary injury as a result of an underlying autoimmune process. PBC is often associated with extrahepatic autoimmune diseases such as Sjögren's syndrome and chronic thyroiditis. Idiopathic thrombocytopenic purpura (ITP) is a well-defined autoimmune disease, but the association of PBC with ITP is rare. However, cases of simultaneous occurrence of PBC and ITP as well as ITP after liver transplantation for PBC have been reported^[1-4]. We describe a case in which ITP developed during the follow-up of PBC in an elderly man and discuss the possible mechanisms underlying the association of the 2 diseases.

CASE REPORT

A 74-year-old man was referred to our hospital in November 2000 because of liver dysfunction detected during a medical checkup. The patient had been diagnosed with nephrotic syndrome in 1995. Laboratory examinations showed elevated serum hepatobiliary enzymes and IgM, and the presence of antimitochondrial antibodies. Serologic markers for Hepatitis B and C viruses were negative. Histopathologic examination of a liver biopsy specimen obtained at laparoscopy revealed non-suppurative destructive cholangitis in the portal area (Figure 1). The diagnosis of PBC (Scheuer stage 3) was confirmed and ursodeoxycholic acid, 900 mg daily, was started. In January and June 2002, the patient underwent endoscopic variceal ligation plus endoscopic injection sclerotherapy as well as argon plasma coagulation for worsening esophageal varices.

In September 2007, the patient was admitted for the

Table 1 Laboratory data on admission

Normal ranges			Normal ranges		
WBC	$4 \times 10^9/L$	3.5-8.5	IgG	2217 mg/dL	870-1700
RBC	$3.44 \times 10^{12}/L$	4.2-5.5	IgA	478 mg/dL	110-410
Hb	10.4 g/dL	13.5-17.0	IgM	217 mg/dL	35-220
Plt	$8 \times 10^9/L$	150-350	CRP	0.11 mg/dL	≤ 0.3
PT-INR	1.14	0.9-1.08	HBsAg	(-)	
APTT	30.6 s	25-40	HCV RNA	(-)	
Fibrinogen	301 mg/dL	178-384	ANA	$\times 640$	
AST	39 IU/L	10-35		Nucleolar	
ALT	21 IU/L	7-42	AMA	$\times 160$	
ALP	390 IU/L	110-360	MPO-ANCA	(+)	
LAP	48 IU/L	30-80	Anti-Jo-1	(-)	
γ GTP	29 IU/L	5-60	Anti-DNA	(-)	
T-Bil	0.5 mg/dL	0.2-1.2	Anti-SSA	(+)	
ChE	144 IU/L	168-470	Anti-SSB	(-)	
T-Chol	107 mg/dL	130-220	Anti-Scl	(-)	
TP	6.8 g/dL	6.5-8.0			
Alb	2.7 g/dL	3.8-5.3			
BUN	14 mg/dL	8.0-20			
Cr	0.92 mg/dL	0.6-1.10	PAIgG	13 200 ng/ 10^7 cells	9-25

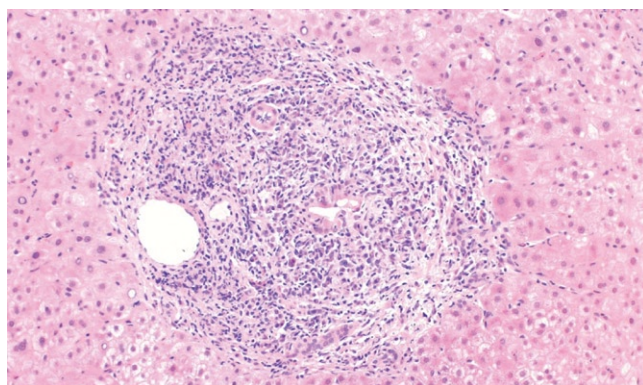


Figure 1 A liver biopsy specimen obtained at laparoscopy showing marked infiltration of lymphocytes and plasma cells, and degeneration of interlobular bile ducts in the portal area (HE, $\times 100$).

treatment of recurrent esophageal varices. The platelet count had ranged between $52 \times 10^9/L$ and $69 \times 10^9/L$ for several years, but it was noted to decrease from $61 \times 10^9/L$ in June 2007 to $8 \times 10^9/L$ just before admission. Before the deterioration of thrombocytopenia, the patient had no infectious diseases and received no other medication. On admission, the patient had neither purpura nor bleeding episodes. Table 1 shows the laboratory data on admission. The platelet-associated IgG level was markedly high. Bone marrow biopsy revealed normocellular marrow without cellular atypia. Ultrasonography and magnetic resonance imaging revealed a cirrhotic liver with splenomegaly, ascites, and gallstones. The spleen size had remained unchanged from previous imaging examinations. Based on these findings, the association of PBC (decompensated liver cirrhosis) with ITP was diagnosed. Human leukocyte antigen (HLA) genotyping determined by polymerase chain reaction-sequencing-based typing or polymerase chain reaction-sequence specific primers (SRL, Inc., Tokyo, Japan) detected A*02010101, B*400201, C*030401, C*07020101, DPB1*0501, DQA1*0103, DQA1*030101,

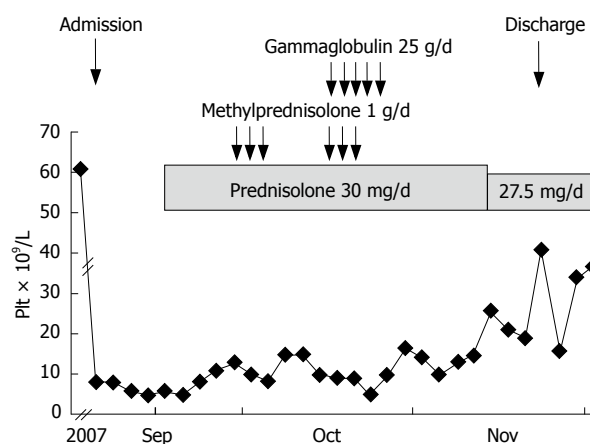


Figure 2 The clinical course of the patient.

DQB1*030201, DQB1*060101, DRB1*080201, and DRB1*080302. The ^{13}C urea breath test for *H. pylori* infection was negative.

Figure 2 shows the clinical course. Oral prednisolone, 30 mg daily, for ITP was started on day 11, and diuretic therapy combined with albumin infusion for ascites was performed. As the platelet count did not increase notably, pulse therapy with intravenous methylprednisolone, 1 g daily, was added on d 22 to 24. However, the response was weak and temporary. On d 31, mild melena was identified. The patient was given a trial of intravenous immune gamma globulin therapy, 25 g daily, on d 32 to 36, combined with a second round of intravenous methylprednisolone pulse therapy on d 32 to 34. Because a moderate response was observed, prednisolone was continued, and the platelet count increased slowly. The ascites was relatively well controlled with diuretics at discharge. Considering the decompensated liver cirrhosis and the platelet count, we determined the patient required careful follow-up of esophageal varices without prophylactic endoscopic therapy.

DISCUSSION

Because immunogenetic factors are believed to influence the development of autoimmune diseases, the relationship between HLA alleles and susceptibility to PBC and ITP has been investigated^[5-8]. In Japanese patients with PBC, the frequency of DPB1*0501, DQA1*0103, DQB1*0601, and DRB1*0803 is increased^[5,6]. On the other hand, those with ITP have an increased frequency of DRB1*0410^[7], and strong associations between anti-platelet surface glycoprotein autoantibodies and HLA alleles have been reported, including an association of anti-glycoprotein IIb/IIIa antibody with DQB1*0401 and DRB1*0405, and of anti-glycoprotein I b/IX antibody with DQB1*0601 and DRB1*0803^[8]. Of these HLA alleles, DPB1*0501, DQA1*0103, DQB1*0601, and DRB1*0803 were detected in the present case. It should be noted that DQB1*0601 and DRB1*0803 are related to both PBC and ITP. The frequency of a combination of the 2 HLA alleles in patients with PBC and ITP is significantly higher than that in general population^[6,8]. Furthermore, it is estimated patients with a combination of the 2 HLA alleles are at least several-fold more susceptible to PBC and ITP. The 2 HLA alleles might be common immunogenetic factors for the development of PBC and ITP, and the mechanism underlying ITP development might be anti-glycoprotein I b/IX antibody-mediated platelet destruction. Unfortunately, anti-glycoprotein autoantibodies were not examined. Immunogenetic analyses should shed light on the mechanism of the association of PBC with ITP.

The present patient already had thrombocytopenia related to liver cirrhosis at the onset of ITP. Based on an evaluation of platelet kinetics, a recent study has shown reduced platelet production and enhanced platelet turnover in patients with liver cirrhosis^[9]. Hypersplenism, as observed in the present case, is well known to enhance platelet turnover; the pathologically enlarged and congested spleen accelerates the sequestration and destruction of platelets. Another mechanism of thrombocytopenia in liver cirrhosis is anti-platelet autoantibody-mediated platelet destruction. Kajihara *et al*^[10] have revealed a similar profile of the anti-glycoprotein IIb/IIIa autoantibody response between patients with liver cirrhosis and those with ITP, and concluded that immune-mediated platelet destruction may contribute, at least in part, to cirrhotic thrombocytopenia. In the present case, an autoimmune response to platelets might have been induced or enhanced by the underlying liver cirrhosis.

Panzer *et al*^[11] have reported autoantibodies eluted from a patient with PBC and ITP precipitate glycoprotein IIb/IIIa of autologous and allogeneic platelets and bind to an epitope of the rat 70-kDa mitochondrial protein M2. Furthermore, computer analysis of published peptide sequences of the mitochondrial protein and glycoprotein IIb/IIIa showed partial amino acid sequence homology, suggesting the possibility of a common antibody-binding site. In another study, it was suggested the development of the immune phenomenon in PBC may also involve immune-mediated platelet destruction^[12]. There might be

a mechanism by which PBC-related autoantibodies cross-reacting with platelet surface autoantigens cause ITP.

In summary, we experienced a rare case of the association of PBC with ITP. HLA genotyping suggests that common immunogenetic factors might be involved in the development of PBC and ITP.

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LETTERS TO THE EDITOR

Non-invasive prediction of oesophageal varices in cirrhosis

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Abstract

Non-invasive predictors of varices in cirrhosis would reduce the need for screening endoscopies. Platelet count and spleen size have been shown to be useful parameters, in mixed groups of cirrhotics with different aetiologies. We evaluated this in two homogeneous groups with cirrhosis due to hepatitis C and alcohol. Non-invasive predictors appear promising in the former group, but less so in the latter group.

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Key words: Varices; Cirrhosis; Endoscopy; Platelet; Spleen

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Sen S, Griffiths WJH. Non-invasive prediction of oesophageal varices in cirrhosis. *World J Gastroenterol* 2008; 14(15): 2454-2455 Available from: URL: <http://www.wjgnet.com/1007-9327/14/2454.asp> DOI: <http://dx.doi.org/10.3748/wjg.14.2454>

TO THE EDITOR

We read with interest the study by Alempijevic *et al* on non-invasive prediction of oesophageal varices in cirrhosis^[1]. We agree this is an important clinical goal, thus reducing the need to screen all individuals with cirrhosis. Importantly, such predictors must have a high sensitivity, even at the cost of a lower specificity, to ensure that patients with varices are not missed. Platelet count and spleen size appear consistently discriminatory in recent

series^[2-6]. Giannini *et al*^[2,3] have suggested the platelet:spleen size ratio as informative. Sharma and Aggarwal^[6] have recently proposed a predictor function derived from these two parameters. Of note, all these studies evaluated mixed groups of cirrhosis with different aetiologies. We routinely measure spleen size by ultrasound and performed a retrospective study of all patients with cirrhosis due to either hepatitis C alone ($n = 93$) or alcohol alone ($n = 77$) who underwent screening gastroscopy in our unit over a four-year period. Patients with tumour, splenectomy or portal vein thrombosis were excluded.

In the hepatitis C group, both platelet count and spleen size had a good predictive ability for the presence of oesophageal varices, while the platelet:spleen size ratio and the reported predictor function were slightly superior (Table 1). The optimum cut-off for the platelet:spleen size ratio was 6.50, compared with 9.09 by Giannini *et al*^[2,3], and that for the predictor function was 1.00, compared with the published figure of 1.09 (although the latter was for large varices only). We found, however, that a simple clinical predictor based on the best cut-off values obtained individually for platelet count and spleen size, was the most sensitive predictor of the presence of oesophageal varices. Platelet count $\leq 90 \times 10^9/L$ and/or spleen size ≥ 14 cm achieved a sensitivity of 92% and a specificity of 69% in this cohort.

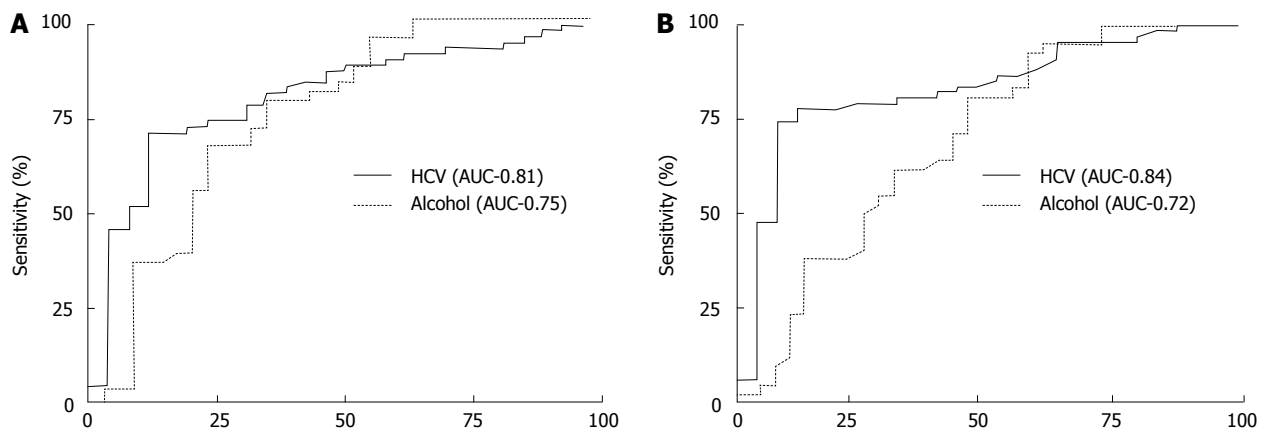
In the alcohol-related cirrhosis group, however, the predictive ability of all these parameters was generally poorer, as evidenced by a lower area under the curve (AUC) as well as a lower sensitivity and specificity compared with hepatitis C (Figure 1 and Table 1). Moreover, for the derived functions, cut-off values, which differed from those noted in the hepatitis C group and from the published values, had to be chosen to get a reasonable balance between sensitivity and specificity. With a platelet count $\leq 120 \times 10^9/L$ and/or spleen size ≥ 12 cm, only a sensitivity of 74% and a specificity of 55% were obtained.

Our data suggest that easily obtainable non-invasive markers are effective for predicting oesophageal varices in hepatitis C cirrhosis but are not as promising for cirrhosis due to other aetiologies such as alcohol. In hepatitis C cirrhosis, portal pressure is presumably relatively stable whereas in alcohol-related liver disease, portal pressure may vary with consumption and abstinence. Furthermore, alcohol can cause thrombocytopenia through folate deficiency and a direct effect on platelet function and survival which may render platelet count a less accurate marker of portal pressure. This may explain why published predictors of varices which incorporate platelet count and

Table 1 Predictive efficacy of platelet count, spleen size and their derived functions in cirrhosis due to hepatitis C and alcohol (mean \pm SD) (AUC-area under curve, ROC-receiver operating characteristic)

	Without varices	With varices	P-value	AUC of ROC curve	Best cut-off predictor value	Sensitivity (%)	Specificity (%)
Hepatitis C cirrhosis	67	26					
Platelet count ($\times 10^9/L$)	136 \pm 53	87 \pm 44	< 0.001	0.78	90	78	61
Spleen size (cm)	12.4 \pm 2.6	15.0 \pm 2.3	< 0.001	0.78	14	82	65
Platelet:Spleen ratio	11.8 \pm 6.0	6.2 \pm 4.3	< 0.001	0.81	6.5	82	65
Platelet and spleen derived predictor ^[6]				0.84	1	79	77
Alcoholic cirrhosis	42	35					
Platelet count ($\times 10^9/L$)	200 \pm 90	142 \pm 99	0.009	0.74	130	79	57
Spleen size (cm)	11.2 \pm 2.4	12.9 \pm 3.5	0.02	0.68	12	71	54
Platelet:Spleen ratio	19.0 \pm 10.6	13.4 \pm 14.2	0.06	0.75	11.1	81	57
Platelet and spleen derived predictor ^[6]				0.72	0.09	71	60

AUC: Area under curve; ROC: Receiver operating characteristic.

**Figure 1** Comparison of receiver operating characteristic (ROC) curves for (A) platelet:spleen size ratio and (B) the derived predictor function^[6] in hepatitis C (HCV) cirrhosis versus alcoholic cirrhosis (AUC-area under the curve).

a mix of aetiologies do not demonstrate the high sensitivity we observed in hepatitis C. The right liver lobe/albumin ratio proposed by Alempijevic *et al*^[1] may, however, prove to be more consistently reliable in generalized cirrhosis. Further prospective studies should help to delineate the optimum approach and we look forward to future consensus guidelines which incorporate these strategies, reducing the burden of endoscopy for patients with cirrhosis.

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 Falk Symposium 163: Chronic Inflammation of Liver and Gut

March 23-26, Seoul, Korea
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 18th Conference of APASL: New Horizons in Hepatology
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 Shanghai - Hong Kong International Liver Congress
www.livercongress.org

April 05-09, Monte-Carlo (Grimaldi Forum), Monaco
 OESO 9th World Congress, The Gastro-esophageal Reflux Disease: from Reflux to Mucosal Inflammation - Management of Adeno- carcinomas
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June 26-28, Bratislava, Slovakia
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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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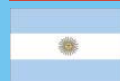
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Treatment of gastrointestinal neuroendocrine tumors with inhibitors of growth factor receptors and their signaling pathways: Recent advances and future perspectives

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Abstract

The limited efficacy of conventional cytotoxic treatment regimes for advanced gastrointestinal neuroendocrine cancers emphasizes the need for novel and more effective medical treatment options. Recent findings on the specific biological features of this family of neoplasms has led to the development of new targeted therapies, which take into account the high vascularization and abundant expression of specific growth factors and cognate tyrosine kinase receptors. This review will briefly summarize the status and future perspectives of antiangiogenic, mTOR- or growth factor receptor-based pharmacological approaches for the innovative treatment of gastrointestinal neuroendocrine tumors. In view of the multitude of novel targeted approaches, the rationale for innovative combination therapies, i.e. combining growth factor (receptor)-targeting agents with chemo- or biotherapeutics or with other novel anticancer drugs such as HDAC or proteasome inhibitors will be taken into account.

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Key words: Growth factor receptor; Neuroendocrine gastrointestinal tumor; Small molecule inhibitor; Monoclonal antibody; Multi kinase inhibition

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INTRODUCTION

Neuroendocrine tumors form a heterogeneous group of malignancies that do not only include gastrointestinal neuroendocrine tumors but also neoplasias such as pheochromocytoma, pituitary tumors, medullary thyroid cancer, and even undifferentiated (small cell) neuroendocrine cancer. Gastroenteropancreatic neuroendocrine tumors (GEP NET) are usually classified according to localization of the primary, grade of differentiation and functionality. The former traditional classification distinguished between pancreatic neuroendocrine tumors and carcinoid tumors^[1]. Both tumor types often display well-differentiated histologic features, and often preserve the ability to release excessive amounts of biogenic amines and/or neuropeptides thereby causing characteristic hypersecretion syndromes. The resulting, often bizarre clinical symptoms are generally well controlled by somatostatin analogs or interferon- α ^[2,3]. However, tumor growth and spread of GEP NETs are not always well controlled by either biotherapy or chemotherapy. Thus, therapeutic options to inhibit growth and spread of gastrointestinal neuroendocrine tumors are still unsatisfactory.

Significant advances in our knowledge of the particular biology of GEP NETs made over the past decades shows that GEP NETs represent a tumor entity with an extraordinary high vascularization along with an abundant production and secretion of growth factors such as VEGF, EGF, IGF, PDGF, HGF, FGF or TGF- α . Expression and signaling of growth factors and their cognate receptors in GEP NETs has been studied quite extensively^[4-10], and paved the way for new and molecular targeted strategies for GEP NET treatment. Among the most promising new therapeutic approaches is the inhibition of synthesis and/or secretion, as well as receptor binding of angiogenic

growth factors such as vascular endothelial growth factor (VEGF). These antiangiogenic approaches are mostly based on the use of specific monoclonal antibodies or tyrosine kinase inhibitors to attenuate tumor microvessel formation and hence the vital supply of the tumor with nutrients and oxygen^[11,12]. Furthermore, dysregulation and/or overexpression of other oncogenic growth factor receptors in GEP NETs, such as the epidermal growth factor receptor (EGFR), insulin-like growth factor receptor-1 (IGF-1R), or the platelet-derived growth factor receptor (mainly PDGFR- β) offer additional targets for future chemotherapeutic intervention^[13,14]. These growth factor receptor-based strategies mainly target the receptors' intrinsic tyrosine kinase activity with small molecule inhibitors or ligand-receptor interactions with monoclonal antibodies. The underlying rationale for this treatment strategy is to specifically interrupt the downstream mitogenic and antiapoptotic signaling cascades that are triggered by ligand-activation of a specific growth factor receptor, events that have been shown to play a crucial role in the expansion and spread of the tumors.

Besides therapeutically targeting growth factor receptors, a third promising approach is the direct inhibition of receptor-mediated downstream signaling pathways such as the phosphatidylinositol-3-kinase (PI3K)-AKT-mammalian target of rapamycin (mTOR) pathway or the Ras/Raf-mitogen-activated kinase (MAPK) pathway^[15,16]. There is increasing evidence that specific inhibition of several key components of these pathways, such as mTOR and Raf, is thought to exert enhanced antineoplastic potency as compared to the single inhibition of just one pathway or pathway-activating receptor. Thus, the abrogation of single growth factor receptor activities are found to be counter-balanced by compensatory signaling and transactivation of other growth factor receptors^[17-20].

This review will provide a perspective overview of selected agents, which are currently in development, consideration or testing for such targeted treatment approaches for GEP NET (Table 1). Moreover, promising approaches, which have not yet been evaluated in GEP NET, but warrant future evaluation, will be discussed.

ANTIANGIOGENIC TREATMENT STRATEGIES

Angiogenesis plays a central role in tumor growth and progression, and its implication has been extensively investigated and described in the literature for various cancers^[21,22]. In the early 1970s, Folkman was the first to develop the concept of angiogenesis-dependent tumor growth and postulated that the specific blocking of blood flow to the tumor should be a promising strategy for cancer treatment^[23].

Among the angiogenic factors/receptors described so far, the vascular endothelial growth factor (VEGF) and VEGF receptor family including the secreted glycoproteins VEGF-A (synonym: VEGF), VEGF-B, VEGF-C, VEGF-D, VEGF-E, the placental growth factors (PlGF-1, -2), and their cognate receptors VEGFR-1 (Flt-1), VEGFR-2 (Flk/KDR) play major

roles (not only in physiological) but also in pathological angiogenesis. VEGF that binds to both VEGFR-1 and -2 is the key regulator of the development of the vascular system and is commonly overexpressed in a variety of solid tumors^[24]. Hypervascularized GEP NETs, have also been demonstrated to (over-)express VEGF and its cognate receptors (VEGFR-1, -2) in the tumor and its surrounding vasculature^[25-28]. In addition, elevated levels of circulating VEGF are correlated with the progression of GEP NETs^[29]. In this line, a recent study confirmed the particular role of VEGF for the prognosis and progression of GEP NET by showing that elevated expression of VEGF correlated with increased angiogenesis and decreased progression-free survival among patients with low-grade neuroendocrine tumors^[30].

Based on the particular significance of VEGF and its receptors (VEGFR-1, -2) in GEP NETs and because of VEGF's action on endothelial cell activation for new tumor vessel formation, the VEGF/VEGFR system has been an extensively studied target for the treatment of GEP NET.

ANTIBODY-BASED ANTIANGIOGENIC THERAPY

Anti-VEGF treatment

Bevacizumab is a humanized murine monoclonal anti-VEGF antibody, which has entered the clinic for antiangiogenic treatment of cancer. Standard cytostatic treatment plus bevacizumab significantly increased survival in metastatic colorectal cancer as compared to standard treatment alone in a phase III clinical trial^[31], a finding that led to the approval of bevacizumab for treatment of colorectal cancer in 2005. Comparable results were obtained in a recent phase III clinical trial with bevacizumab for treatment of non-small cell lung cancer (NSCLC). This study was interrupted before finalization because of the obvious survival advantage of patients in the bevacizumab arm^[32].

The first clinical trials with bevacizumab for treatment of GEP NET were reported 3 years ago^[33]. In a randomized Phase II trial, the effect of monotherapy with bevacizumab (15 mg/kg every 3 wk) was compared to the effects of pegylated IFN- α_{2b} (0.5 μ g/kg per week) in patients with advanced carcinoid tumors. After 18 wk an almost 30% higher rate of progression free survival rate (PFS) was observed in the bevacizumab arm (98% PFS) as compared to the peg-IFN- α_{2b} arm (68% PSF) (NIH: NCT00055809). In bevacizumab-treated patients, CT scan, monitoring the antiangiogenic effects of bevacizumab at the individual patients' level, showed a dramatic decrease in tumor perfusion. Based on these encouraging findings a phase III trial is currently being proposed to evaluate the benefit of bevacizumab as compared to IFN- α_{2b} for the treatment of patients with advanced carcinoid tumors and a poor-prognosis who are under stable doses of depot octreotide (South West Oncology Group; unpublished study-protocol of study: S0518).

Several other studies using bevacizumab for combination therapy of GEP NET are currently ongoing.

Table 1 Current status of clinical trials with agents that target growth factor receptors and related signaling pathways for treatment of gastrointestinal neuroendocrine tumors

Name	Target	Mechanism	Tumor type	Cotreatment	Status	Reference
Bevacizumab	VEGF	VEGF-neutralizing antibody	Carcinoid	Peg IFN- α	Phase II	[35]
			Carcinoid	Depot-octreotide	Phase III	South West Oncology Group: S50518
			Carcinoid	Panzem	Phase II	NCT00227617
			Advanced GEP NET	FOLFOX	Phase I / II	NCT00328497
			Advanced GEP NET	Oxaliplatin, capecitabine	Phase II	NCT00398320
Sunitinib	VEGFR, PDGFR, c-KIT, FLT3	Tyrosine kinase inhibitor	Pancreatic and other unresectable carcinoid	Temzolomide	Phase II	NCT00137774
			Carcinoid		Phase II	[48]
			Carcinoid		Phase II	NCT00428597
			Carcinoid ¹		Phase II	NCT00434109
			Low-, intermediate grade GEP NET		Phase II	NCT00454363
Pazopanib	Pan-VEGFR, PDGFR, c-KIT	Tyrosine kinase inhibitor	Low-grade GEP NET		Phase II	NCT00427349
AMG706	pan-VEGFR, PDGFR, c-KIT	Tyrosine kinase inhibitor	Low-grade GEP NET		Phase II	NCT00427349
Vatalanib	VEGFR, PDGFR, c-KIT	Tyrosine kinase inhibitor	Progressive GEP NET ²		Phase II	[64]
Gefitinib	EGFR	Tyrosine kinase inhibitor	Progressive GEP NET		Phase II ³	NCT00227773
			Progressive GEP NET	Cetuximab	Phase II	[79]
NVP-AEW541	IGF-1R	Tyrosine kinase inhibitor	Advanced GEP NET		Phase II	NCT00397384
Everolimus	mTOR	Protein kinase inhibitor	NET cells		Pre-clinical	[14]
			Islet carcinoid		Phase II	[136]
Temsirolimus	mTOR	Protein kinase inhibitor	Carcinoid	Octreotide	Phase II	[127]
			Recurrent, metastatic GEP NET		Phase II	[137]
Sorafenib	c-Raf, B-Raf, VEGFR, PDGFR	Tyrosine kinase inhibitor	Progressive, metastatic GEP NET		Phase II	NCT00131911
Imatinib	PDGFR, c-KIT, ABL	Tyrosine kinase inhibitor	Carcinoid		Phase II	[127]
Bortezomib	Proteasome	Proteasome inhibitor	Advanced GEP NET		Phase II	[59]
			Metastatic GEP NET		Phase II	[153]

¹Liver predominant metastases after hepatic arterial embolization; ²Progressive after somatostatin treatment; ³Withdrawn.

A pending Phase I / II trial is recruiting patients with advanced neuroendocrine tumors to determine the safety and efficacy of bevacizumab in combination with 5-fluorouracil, leucovorin and oxaliplatin (FOLFOX) (NIH: NCT00227617). Additionally, the efficacy of bevacizumab together with oxaliplatin and capecitabine is currently being investigated in metastatic and unresectable GEP NETs in a non-randomized, open label phase II trial (NIH: NCT00398320). Bevacizumab is also being tested in combination with 2-methoxyestradiol (Panzem) in patients with locally advanced or metastatic carcinoid tumors (NIH: NCT00328497). Panzem is a metabolite of estradiol that has recently emerged as a promising anticancer agent because of its potent growth-inhibitory and proapoptotic effects on both endothelial and tumor cells^[34,35]. Besides other antiangiogenic and cytotoxic properties panzem's mode of action involves the inhibition of the hypoxia-inducible factor (HIF)-1 α , a transcription factor, which drives the expression of several pro-angiogenic genes^[36]. *In vitro* and *in vivo* (animal) studies of several tumor types, including sarcoma, lung, and breast cancer, have documented potent inhibitory effects on tumor cells and angiogenesis without major clinical signs of toxicity^[37]. Thus, a dual targeting of GEP NETs at the level of both the tumor cell and tumor microvessel formation

by panzem and bevacizumab appears to be a promising combination.

Another interesting phase II trial currently explores the combination of bevacizumab and the DNA-methylating drug temzolomide (NIH: NCT00137774). The rationale for this particular combination is based on findings of a former phase II trial, in which an enhanced antitumoral efficacy of a combination treatment with temzolomide together with the mildly antiangiogenic VEGFR- and bFGFR- inhibitor, thalidomide, was demonstrated for advanced pancreatic GEP NETs and metastatic carcinoid tumors^[38]. However, thalidomide is considered a risky drug that has been associated with neurological side effects and severe and frequent teratogenicity in the 1950s and 1960s^[39]. Thus, in order to replace thalidomide by a safer antiangiogenic drug, bevacizumab is now being studied as a combination partner for temzolomide.

Anti-PIGF treatment

The use of a neutralizing anti-PIGF monoclonal antibody in VEGF-inhibitor resistant tumors is an attractive new antiangiogenic that has been tested in an animal study^[40]. The antibody specifically inhibits the binding of PIGF to its receptor VEGFR-1, present on tumor associated endothelial cells and macrophages. The underlying idea of

using this approach derives from gene inactivation studies showing that endogenous PlGF is redundant for vascular development and physiological vessel maintenance, but an important contributor to the “angiogenic switch” in solid tumor growth. This lead to the hypothesis that unlike VEGF inhibitors, PlGF inhibition might reduce pathological angiogenesis, without affecting physiological blood vessel homeostasis and thus not causing unwanted side effects. Hence, anti-PlGF treatment could perhaps substitute for anti-VEGF therapy in the future. Moreover, as PlGF levels are known to increase in the circulation of cancer patients receiving anti-VEGF treatment^[41-43], anti-PlGF could also counter this potential downside of anti-VEGF therapy. In this line, the data on inhibition of angiogenesis, lymphangiogenesis, tumor growth and motility in the anti-PlGF-treated anti-VEGF-resistant tumor bearing mice are impressive, especially with regard to blocking the so-called rescue-angiogenesis, a major problem in current antiangiogenic approaches, together with an excellent tolerability compatibility of the treatment. In addition, anti-PlGF treatment may permit long-term treatment of cancers in children, pregnant women, or patients at risk for thrombotic, cardiac or other complications for whom the adverse effects of other VEGF/VEGFR-inhibitors may be excessive and prohibitive.

Antiangiogenic therapy with small molecule inhibitors

In addition, several agents, which inhibit the tyrosine kinase activity of angiogenic growth factor receptors like the VEGFR or PDGFR, have been synthesized by combinatorial chemistry. These tyrosine kinase inhibitors are small molecules that occupy the ATP binding site of the tyrosine kinase domain of the intracellular portion of the receptor. Because of their effects on downstream signaling, these inhibitors interfere with a number of key biologic functions associated with VEGFR activation. Although drugs that are directed to the VEGFR proved their clinical efficacy, the redundancy in the angiogenesis pathways will likely necessitate multiple targeting agents appealing^[44].

SUNITINIB

Recent clinical studies showed remarkable growth suppression of several non-GEP NET tumors by sunitinib, an orally available inhibitor of multiple receptor tyrosine kinases such as VEGFR-, PDGF- β R, c-KIT and FLT-3. Sunitinib has been approved for the treatment of renal cell carcinoma^[45]. With restricted indication sunitinib is also approved for the therapy of gastrointestinal stromal tumors (GIST)^[46] and is currently tested in phase I and II trials for hepatocellular carcinoma (NIH: NCT00361309; NCT00247676).

In GEP NETs, a phase II trial reported partial responses of 15% in pancreatic islet cell carcinoma and 2% in carcinoid tumors. In both groups, a high rate of disease stabilization, 75% for islet tumors and 93% for carcinoid tumors, was observed^[47,48]. Based on these encouraging results an international randomized and

double-blind phase III trial has been launched to study the effect of sunitinib given daily as a continuous dose versus placebo in patients with advanced carcinoids and islet cell tumors (NIH: NCT00428597). In a single-center, non-randomized, prospective phase II trial GEP NET patients with liver-predominant metastases are currently recruited to investigate sunitinib efficacy to improve time to liver cancer progression following hepatic arterial embolization (NIH: NCT00434109).

PAZOPANIB AND AMG706

These two orally available drugs are pan-VEGFR inhibitors, which also block the activity of the PDGFR and c-kit. Antineoplastic activity of AMG706 has been shown in preclinical non-NET models^[49]. Good tolerability and antitumor efficacy have been observed in first clinical trials with advanced refractory solid tumors^[42]. Additional studies of AMG706 as monotherapy and in combination with various agents are ongoing^[42,50]. At present, a clinical study assesses the efficacy of a monotherapy with AMG706 in patients with low-grade NET (NIH: NCT00427349). The primary goal of the trial is to evaluate the tolerability and 4-mo progression free survival under AMG706 treatment.

For pazopanib (GW786034) an excellent antiangiogenic effect on both tumor cells and tumor associated endothelial cells has been shown in pre- and early clinical studies, and good tolerability has been reported in patients with ovarian- and advanced renal cell carcinoma^[51,52]. Currently a phase II trials is recruiting patients to evaluate the suitability of pazopanib for the treatment of advanced low-grade or intermediate-grade NET (NIH: NCT00454363).

IMATINIB

The phenylaminopyrimidine derivative Imatinib mesylate (Gleevec) is an orally available small molecule that selectively inhibits the tyrosine kinases ABL, c-Kit and PDGFR. Due to its ABL- and c-Kit-inhibiting potency, imatinib has significantly improved the treatment of cancers that crucially depend on the activation of these growth factor receptors, such as chronic myelogenous leukemia and gastrointestinal stromal tumors^[53,54]. Moreover, imatinib demonstrated clinical efficacy by inhibition of PDGFR-signaling in dermatofibrosarcoma protuberans, a neoplasm that depends on an abnormal activation of PDGFR β through an autocrine loop^[55].

Although there have been no reported mutations in ABL, c-Kit, and PDGFR in NET, they are characterized by a simultaneous upregulation of PDGF ligands and their receptors (PDGFR- α and PDGFR- β)^[6,56,57]. Thus, imatinib may also be interesting for GEP NET treatment. Yao and coworkers evaluated this hypothesis in a phase II trial of patients with advanced carcinoids who were treated with 400mg imatinib twice daily. However, only one of the 27 treated patients achieved an objective response, while 17 patients had stable disease, and 9 patients showed disease progression when evaluated with RECIST criteria (Response Evaluation Criteria in Solid Tumors)^[58]. Another

study that included 15 patients with advanced GEP NET, imatinib (400 mg or 800 mg imatinib/day for up to 12 mo) was not effective, but was associated with remarkable toxicity and increased bleeding tendency^[59].

The low response rate to imatinib, as compared to other antiangiogenic agents, may be related to its sole activity towards the PDGFR, while it completely lacks VEGFR-inhibitory activity. Moreover, PDGFR-inhibition by imatinib is relatively low, as compared to other PDGFR-targeting agents. For instance, the small molecule inhibitor sunitinib has a tenfold higher potency to inhibit PDGFR-signaling, and moreover also inhibits VEGFR-signaling.

From the data obtained so far, monotherapy with imatinib does not seem to be beneficial for patients with advanced GEP NET. Future trials will have to focus on imatinib's suitability as an additive agent for combination therapy, e.g. in conjunction with VEGFR-inhibitors^[60].

VATALANIB

Vatalanib (PTK787/ZK222584) inhibits the activities of VEGFR-1 and -2 tyrosine kinases and shows antineoplastic effects in several solid tumors^[61-63]. This oral agent achieved a 25% biochemical partial response rate (defined as > 50% decrease in 5-HIAA) in NET patients with progressive disease following unsuccessful somatostatin-analog therapy^[64]. Although partial radiographic responses have not been observed, further recruitment for this phase II trial is currently ongoing. By contrast, another phase II trial investigating vatalanib alone and in combination with somatostatin analogs for the treatment of progressive NET has only recently been withdrawn (NIH: NCT00227773). At present, it is unclear if this was due to insufficient antitumoral activity, toxicity or other reasons.

EGFR-BASED STRATEGIES

The crucial role of epidermal growth factor receptor (EGFR) in tumor proliferation and its overexpression in several solid tumors have provided the rationale for targeting and interrupting this key signaling network. EGFR blockade with monoclonal antibodies and tyrosine kinase inhibitors has translated into clinical benefit in gastrointestinal tumors, particularly colorectal cancer^[65].

Over the past few years, three EGFR-specific agents have received regulatory approval: (1) The monoclonal anti-EGFR antibody cetuximab for metastatic colorectal cancer, and squamous cell carcinoma of the head and neck; (2) The tyrosine kinase inhibitor erlotinib for advanced or metastatic pancreatic cancer and NSCLC; and (3) The EGFR tyrosine kinase inhibitor gefitinib for advanced or metastatic NSCLC. However, the general FDA approval for NSCLC treatment with gefitinib was recently withdrawn after it failed to demonstrate a survival benefit either alone or with chemotherapy in three phase III trials^[66,67].

Several reports indicate that EGFRs are frequently expressed and upregulated in NET in general^[68,69], as well as in gastrointestinal NET^[70-75]. In addition, EGFR contributes to the growth characteristics of GEP NETs^[76-78]. Hence, the EGFR is an attractive target for

GEP NET disease, and EGFR-inhibitors have already been shown to inhibit GEP NET cell growth *in vitro*^[13].

Despite the encouraging preliminary findings on the general suitability of anti-EGFR-based approaches for the treatment of GEP NET^[13,78], no clinical trials have been conducted so far, while their efficacy has been demonstrated in other tumor entities, especially colorectal cancer, renal cell carcinoma and NSCLC.

Hobday and coworkers now conducted a phase II trial of gefitinib monotherapy in patients with progressive GEP NET. The study showed that gefitinib is well tolerated and prolongs disease stabilization in patients with prior documented objective progression of islet cell carcinoma and carcinoid tumors. The 6-mo progression free survival of gefitinib-treated patients was 30% for carcinoid tumors and 10% for islet cell carcinoma. However, no objective responses have been observed^[79].

In another ongoing trial the efficacy of a combination treatment with the EGFR-TK inhibitor, erlotinib^[80], together with the EGFR-antibody, cetuximab^[81], is currently evaluated in patients with advanced gastrointestinal cancers, including carcinoid tumors (NIH: NCT00397384). The rationale for the combination of two EGFR-targeted agents is that erlotinib may stop the growth of GEP NET cells by blocking essential growth-related signaling pathways, while cetuximab may additionally mark GEP NET cells with IgG for attack by immune effector cells^[82]. Moreover, erlotinib and cetuximab are thought to stop the growth of GEP NET cells also by antiangiogenic effects on the tumor endothelium.

Anti-EGFR-based therapies have their greatest potential in combination either with conventional cytostatics or with other targeted-agents^[81,83-85]. Again, the rationale for using combination therapies is the existence of multilevel receptor cross-stimulation or of redundant signaling pathways that lead to neoplasia. Blocking only one of these pathways allows others to act as salvage or escape mechanisms for cancer cells. Preclinical evidence of synergistic antitumor activity achievable by combining targeted agents that block multiple signaling pathways has recently emerged^[17,86]. The multi-target approach can be accomplished by using either combinations of selective agents or single agents, which interfere with various targets^[18].

IGF/IGFR-BASED STRATEGIES

Both insulin-like growth factors, IGF- I and - II, and their receptor tyrosine kinase, IGF-1R, are involved in the development and progression of cancer^[87-90]. Activation of the IGF-1R by IGF- I and - II plays a pivotal role in tumor cell proliferation and spread, by promoting cell cycle progression, preventing apoptosis, and by regulating and maintaining the metastatic tumor phenotype. A wide variety of tumors including GEP NET show abnormal or enhanced expression of IGFs and IGF-1R, which leads to auto- and paracrine growth stimulation, and which has been correlated with enhanced proliferation, tumor de-differentiation, disease stage, development of metastases and reduced patient survival. In GEP NET, the dysregulation of the IGF/IGFR system also contributes

to the excessive secretion of biogenic amines^[8,91-95]. In gastrinoma patients, the increased expression of the IGF/IGFR-system is associated with low curability and the development of metastases^[96,97]. Thus, the inhibition of the functionally upregulated IGF/IGFR-signaling system is a promising novel approach to treat GEP NET.

Several groups have demonstrated the therapeutic potential of interfering with IGF-1R mediated signaling *in vitro* and *in vivo*, including the use of IGF-1R blocking antibodies, IGF-1R antisense oligonucleotides, or IGF-1R siRNA^[98-101]. Recently, we and others validated the potent and selective IGF-1R tyrosine kinase inhibitor NVP-AEW541 as a promising novel agent for the therapy of several cancers^[102-104], including GEP NET^[14]. The antineoplastic properties of NVP-AEW541 and related compounds such as NVP-ADW742 have been demonstrated in preclinical studies on Ewing's sarcoma-bearing mice, fibrosarcoma, breast cancer, and musculoskeletal carcinoma^[105-109]. Specific IGFR-antibodies potently suppressed prostate and breast cancer cell growth *in vitro*^[110]. The clinically most advanced anti-IGFR antibody is CP-751 871, which is currently being tested in three phase II trials for advanced breast cancer, NSCLC and prostate cancer (www.clinical-trials.gov). Importantly, the preliminary clinical studies indicate that IGFR-inhibition is well tolerated^[106,111,112]. Safety is important, since IGFR-based inhibition has long been regarded as a high-risk intervention, because of the high homology of the IGF-1R receptor with the related insulin-receptor, and the fear that IGF-1R tyrosine kinase inhibitors may lead to insulin resistance and overt diabetes^[113]. However, the current *in vivo* data do not support this assumption, resulting in a growing interest in anti-IGFR-based therapies^[114].

Due to crosstalk between the signaling of the IGF/IGFR system and other growth factor receptors that can attenuate the antineoplastic effect of monotherapeutic approaches, IGF/IGFR-targeting therapies will likely have to be combined with other therapies to enhance efficacy^[115,116]. This can be achieved by dual-targeting the EGFR- and the IGF-1R, since the EGFR is activated by the IGF/IGFR-system leading to mito-oncogenic EGFR-tyrosine kinase activity without ligand stimulation of the EGFR^[20]. In this line IGFR- combined with EGFR-inhibition can over-additively enhance the antineoplastic effect of the respective monotherapies in gastrointestinal cancers^[81,83,104].

DUAL-TARGETING SMALL MOLECULE INHIBITORS

The use of dual-targeting small molecule inhibitors, simultaneously blocking less related kinases such as VEGFR and EGFR tyrosine kinases, may also become promising for future treatment of GEP NET. These agents inhibit both tumor cell proliferation/survival by blocking mito-oncogenic EGFR signaling of the tumor cells and angiogenesis by inhibiting endothelial VEGFRs. In recent *in vivo* studies of non-GEP NET tumor models (colon, cholangiocarcinoma, prostate, NSCLC) the

dual-targeting tyrosine kinase inhibitor NVP-AEE788 displayed significant antineoplastic efficacy^[117-120]. Also for ZD6474 (zactima), another EGFR/VEGFR tyrosine kinase inhibitor, promising phase II/III results were reported for NSCLC and thyroid cancer showing response rates of 30% in patients with locally advanced medullary thyroid cancer^[121] as well as significant prolongation in the progressive free survival of NSCLC patients^[122,123]. Furthermore, dual targeting of the EGFR and the insulin like growth factor receptor are promising new approaches for the treatment of solid tumors, including the GEP NETs^[116].

OTHER STRATEGIES

Targeting the 'mammalian target of rapamycin' (mTOR) pathway

The activated PI3K/AKT/mTOR pathway has emerged as a novel contributor to (GEP NET-) tumor development. PI3K associates with the intracellular domain of several growth factor receptors. Upon receptor activation, PI3K triggers the generation of phosphatidylinositol 3,4,5-trisphosphate (PIP3), which provokes the subsequent activation of AKT, a serine/threonine kinase that activates multiple cellular target proteins, such as the mammalian target of rapamycin (mTOR) subfamily. mTOR is a serine-threonine kinase that regulates apoptosis, proliferation and cell growth by modulating cell cycle progression. Specifically, mTOR is involved in the modulation of mRNA-translation of proteins, which are necessary for cell cycle progression from G1 to S-phase, including the E4-binding protein (E4-BP1), and p70^{S6} kinase^[124].

In nontransformed cells the PI3K/AKT/mTOR pathway is controlled by the phosphatase and tensin homolog deleted on chromosome ten (PTEN), a tumor suppressor which inhibits this pathway by reversing PI3K and subsequent AKT activation. Mutation or silencing of the PTEN gene leads to activation of the mTOR pathway and promotes carcinogenesis. Loss of PTEN expression has been shown in GEP NET^[125]. Accordingly, in sporadic islet cell carcinoma a frequent loss of 10q, the site of the PTEN gene, as well as an altered subcellular localization of PTEN have been reported^[126,127]. Thus, constitutive activation of the PI3K/AKT/mTOR pathway can be due to enhanced stimulation of growth factor receptors, like EGFR and IGFR, but also to decreased PTEN expression or to its altered cellular compartmentalization^[128]. Importantly, 76% of all GEP NETs display constitutive AKT phosphorylation^[78,125]. It is therefore likely that a majority of GEP NET is sensitive to mTOR inhibitors. Indeed, antiproliferative effects of mTOR inhibition in GEP NET cells were recently demonstrated *in vitro*^[129].

mTOR-inhibitors

The natural antibiotic rapamycin (sirolimus) is a potent inhibitor of mTOR^[130]. Recently, three analogues of rapamycin with superior pharmacokinetic and biological properties have emerged. The cell cycle inhibitor-779 (CCI-779, temsirolimus) is a soluble ester analogue. RAD001 [40-O-(2-hydroxyethyl)-rapamycin, everolimus] is

a derivative of rapamycin with high oral bioavailability, and AP23573 is a non-pro-drug analogue of rapamycin. These agents have been tested successfully for their antineoplastic potency and/or tolerability in various malignancies in early clinical trials (e.g. CCI-779 in renal, breast and lung cancers), or are currently being studied in open clinical trials for the treatment of colorectal, endometrial, and brain tumors (RAD001, everolimus)^[131-133]. AP23573 has been successfully tested in a phase II trial in sarcomas^[134] and two phase I studies in patients with refractory or advanced solid tumors showed partial responses and disease stabilization in individual patients^[135].

So far, two-phase II trials exploring mTOR-inhibitors for NET treatment have been reported. Studies with everolimus (RAD001)^[136] and temsirolimus (CCI-779)^[137] have recently completed the recruitment of low-grade NET patients.

The study by Yao *et al.*, reported on 32 patients (18 carcinoids, 13 islet cell carcinomas) who had received 5 mg everolimus orally per day and depot octreotide 30 mg intramuscularly every 28 d. After 12 wk of treatment, the evaluation by RECIST criteria (response evaluation criteria in solid tumors) showed a 15% response. There were four patients with partial response, 22 with stable disease and 4 patients with progressive disease. Progression occurred in two carcinoids and two islet-cell carcinomas. The rate of progression free survival (PFS) at wk 24 was 64% and the treatment was generally well-tolerated^[136]. The promising results of this study led to the development of a multicenter phase II study, RADIANT (RAD001 in advanced NET), in which everolimus (RAD001) is investigated as second-line treatment in patients with advanced pancreatic neuroendocrine tumors after failure of cytotoxic chemotherapy. A similar phase II trial has been activated to evaluate RAD001 in patients with carcinoid tumors^[127]. In both cases, RAD001 will be used as a monotherapy and in combination with octreotide, which inhibits the activated IGF/IGFR pathway of GEP NET. The underlying rationale is that the dual inhibition of mTOR activation by directly targeting mTOR with everolimus, and also its upstream activation *via* IGF/IGFR-signaling with octreotide will completely abrogate this important pathway in NET. This assumption is supported by *in vitro* data that demonstrated the antiproliferative potency of the dual inhibition of endogenous and IGF-stimulated mTOR activity of GEP NET cells by rapamycin^[8].

In the other phase II trial^[137], the clinical and pharmacodynamic effects of the temsirolimus, were investigated in 37 patients with recurrent or metastatic GEP NET. However, temsirolimus showed only modest activity accompanied by distinct but manageable drug-related adverse effects (mainly fatigue, hyperglycemia, rash). The authors concluded that based on the sobering results of this study no further investigation of temsirolimus as a single agent in patients with advanced GEP NET was justifiable. However, evaluation of temsirolimus in combination with other targeted agents, such as multi-kinase inhibitors or antiangiogenic compounds, was suggested^[124].

Targeting the Ras/Raf/MAPK pathway

The proliferative Ras/Raf/MEK/ERK pathway is one of the key signaling cascades that underlie the development and maintenance of cancers. This pathway transduces extracellular signals from the various growth factor receptor tyrosine kinases (e.g. EGFR, IGFR, VEGFR, PDGFR) to the nucleus with a series of specific phosphorylation events, resulting in the expression of proteins for cell cycle progression, apoptosis resistance, extracellular matrix remodeling, cellular motility, angiogenesis or drug resistance^[138]. Dysregulation of this crucial pathway occurs due to oncogenic transformation of Ras and Raf isoforms, or to overexpression and/or overactivation (*via* phosphorylation) of the Ras and Raf genes^[139,140]. Although activating mutations of (B)-Raf are rare in GEP NET^[141], wildtype B-RAF and its activating small G-protein Rap-1 are highly prevalent in the majority of GEP NET. Overexpression of Rap-1 was shown to activate MAPK-signaling and the expression of mitogenic transcription factors of GEP NET cells, thus providing an interesting molecular target for GEP NET treatment^[142].

SORAFENIB

The bi-aryl urea derivative sorafenib (nexavarTM) is an oral multi-kinase inhibitor, which targets kinases of wild-type B-Raf, mutant V559EB-Raf and C-Raf, and importantly receptor tyrosine kinases involved in angiogenesis, including VEGFR-2, and -3, and PDGFR^[143]. Sorafenib has been approved by the FDA for the treatment of advanced renal cell carcinoma, and only recently it gained accelerated approval for the treatment of inoperable hepatocellular cancer.

Sorafenib's effect on several molecular targets in addition to the Raf isoforms makes it difficult to determine which of its targets contributes most to the anti-tumor activity of sorafenib in the particular tumor types. For instance, a recent HCC trial suggested that inhibition of the Raf/MEK/ERK pathway was central to sorafenib's mode of anti-tumor action^[144], whereas in other cancers, such as renal cell carcinoma or NSCLC the antineoplastic activity was attributed mainly to its antiangiogenic activity^[16,145]. In 2005, an international multicenter phase II trial has started to evaluate the efficacy of sorafenib in patients with progressive metastatic NET (NIH: NCT00131911). Results from this study, which enrolled 90 patients, are pending.

Targeting the proteasome

Effective cancer treatment may also be achieved by inhibition of the 26S proteasome, a large protease complex that is present in both the nucleus and the cytoplasm of eukaryotic cells. The proteasome functions as a proof-reader and terminator of proteins branded for destruction by the attachment of ubiquitin. The so-called ubiquitin-proteasome pathway (UPP) is the major non-lysosomal proteolytic system in eukaryotic cells and triggers degradation of a multitude of proteins, including those involved in cell cycle progression, apoptosis, nuclear factor kappaB (NF-κB) activation, and angiogenesis, as well as

mutant, damaged, and misfolded proteins^[146]. Inhibition of the proteasome has emerged as an attractive target for cancer therapy, since a functional UPP is critical for cell survival and proliferation, especially of cancer cells.

BORTEZOMIB

Bortezomib (Velcade™) blocks multi-ubiquitinated protein degradation by inhibiting the active site threonine residue of the 26S proteasome in a competitive and reversible manner^[147]. Antineoplastic activity of bortezomib has been documented in several *in vitro* and *in vivo* studies^[148-150], including NET cells^[151]. Bortezomib is the first proteasome inhibitor that has been approved for treatment of advanced multiple myeloma and mantle cell lymphoma^[146,152].

So far, only one clinical study on bortezomib in advanced metastatic GEP NET has been reported. However, in contrast to the encouraging findings in other cancers, no or only marginal responses to bortezomib monotherapy was observed in the investigated 12 carcinoid and 4 islet carcinoma patients. Given the slow growing nature of these tumors, the observed disease stabilization of 69% (11 of 16 patients) could not be attributed unequivocally to an antitumor effect of bortezomib. Although bortezomib was generally well-tolerated, peripheral sensory neuropathy developed in 37% of the patients^[153]. Specific attention has to be paid to such side effects, when bortezomib (or other targeted agents) are to be combined with other antitumoral drugs, especially conventional chemotherapy, which likely increases gastrointestinal or neurologic toxicity.

Moreover, bortezomib has been combined with multi-kinase inhibitors or histone deacetylase inhibitors (HDAC). Especially, the combination of bortezomib with HDAC inhibitors appears to be a promising approach in GEP NET disease. Baradari and coworkers showed that HDAC inhibition had strong antiproliferative and proapoptotic effects in GEP NET cells^[154]. Recently, the potency of bortezomib combined with HDAC inhibitors has been demonstrated for other gastrointestinal tumors, too. Thus, HDAC inhibition by the benzamide derivative, MS-275 combined with bortezomib led to an overadditive growth inhibition of cholangiocarcinoma cells^[148]. Hence, targeting two or more molecular pathways at the same time appears promising for innovative treatment strategies of GEP NET disease.

CONCLUSION

Targeted-therapies, which specifically inhibit growth factor receptors and their related signaling pathways are promising approaches for the innovative medical treatment of GEP NET disease. Especially antiangiogenic strategies, multi-kinase or mTOR inhibition as well as combination treatments with biotherapeutics or cytostatics emerge to prove particularly efficient, as they leave fewer mechanisms of escape for the tumor cells. Combinations of these targeted drugs are particularly intriguing, and in the future agents like the multi-kinase inhibitors sunitinib or sorafenib

as well as mTOR inhibitors will be combined with other growth factor receptor inhibitors, histone deacetylase inhibitors, proteasome inhibitors, biotherapeutics or cytostatics to effectively control advanced GEP NET. The advantage of such novel combination therapies is their higher tumor cell specificity and higher efficacy, combined with acceptable toxicity and side effects. The novel combination treatments will widen the therapeutic spectrum for GEP NET; the results of (ongoing) clinical studies are eagerly awaited.

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REVIEW

Nonalcoholic fatty liver disease: An overview of current insights in pathogenesis, diagnosis and treatment

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Abstract

Estimates of people suffering from overweight (one billion) and obesity (300 million) are increasing. The accumulation of triglycerides in the liver, in the absence of excess alcohol intake, has been described in the early sixties. It was not until 1980, however, that Ludwig *et al* named this condition nonalcoholic steatohepatitis (NASH). Subsequently, nonalcoholic fatty liver disease (NAFLD) has been used as a general name for conditions ranging from simple steatosis through steatohepatitis to end-stage liver disease (cirrhosis). Many studies have demonstrated the significant correlation with obesity and insulin resistance. Other studies have revealed a significant correlation between hepatic steatosis, cardiovascular disease and increased intima-media thickness. WHO estimated that at least two million patients will develop cirrhosis due to hepatic steatosis in the years to come. Longitudinal cohort studies have demonstrated that those patients with cirrhosis have a similar risk to develop hepatocellular carcinoma as those with other causes of cirrhosis. Taken all together, NAFLD has become the third most important indication for liver transplantation. Therefore, training programmes in internal medicine, gastroenterology and hepatology should stress the importance of diagnosing this entity and treat properly those at risk for developing complications of portal hypertension and concomitant cardiovascular disease. This review will focus on the clinical characteristics, pathophysiology, imaging techniques and the readily available therapeutic options.

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Key words: Non-alcoholic fatty liver disease; Non-alcoholic steatohepatitis; Insulin resistance; Liver; Obesity; Steatosis

INTRODUCTION

When living creatures have a surplus of food at their disposition, they start to hoard energy for future scarce times. The breakthrough in agriculture and founding of villages around 9500 BC in several independent parts of the world not only laid the pavement for modern society, but also gave people the opportunity to cultivate and store more food than needed for their support and their families. This excess of energy has led to the current situation nowadays where the caloric availability exceeds the caloric needs by far. The human body still possesses the quality to store excess energy in adipocytes, a quality that now works in its disadvantage. The extreme storage of excess energy and a reduction in physical activity have led to a world-wide epidemic of obesity. The World Health Organization (WHO) estimated that the number of overweight (BMI > 25) individuals is over one billion, of whom 300 million are obese (BMI > 30)^[1]. In future prospects, this number will increase further^[2,3]. Although the biggest concern of most obese or overweight persons is about their appearance, obesity has been recognised as a pathogenic factor for a vast array of other pathologies, such as Diabetes mellitus, Insulin resistance, Hypertension, Dyslipidemia, Endocrine changes, Kidney stones, Cancer (overall and specific), Coronary disease, Heart failure, Myocardial steatosis, Atrial fibrillation/flutter, Stroke, Venous thrombosis, Hepatobiliary disease, GERD/esophageal cancer, Osteoarthritis, Skin changes, Gout, Dementia, Psychosocial *etc*. A subset of diseases often occurring in the same patient have been clustered, now known as the metabolic syndrome^[4-6]. Vehement research has unravelled major parts of the pathophysiological mechanisms underlying obesity and metabolic syndrome, although many issues have not been explained yet^[7,8]. It has been shown that non-alcoholic fatty liver disease (NAFLD) has a strong relation with metabolic syndrome^[9-16]. NAFLD mainly with accumula-

tion of triglycerides in the liver, in the absence of excess alcohol intake, has been described in the early sixties^[17,18]. It was not until 1980, however, that Ludwig *et al* named this condition non-alcoholic steatohepatitis (NASH)^[19]. Subsequently, NAFLD has been used as a general name for conditions ranging from simple steatosis through steatohepatitis to end-stage liver disease (cirrhosis)^[20]. The first is rather benign^[21,22], the second is of significant clinical importance^[23] and the last one has an increased risk of hepatocellular carcinoma^[24,25]. An almost universal association with hepatic and adipose tissue insulin resistance (IR) has been established in a number of studies^[26-34]. Browning *et al*^[35] used ¹H-NMR-spectroscopy to measure the hepatic triglyceride content in a multi-ethnic urban US population-based study. Hepatic steatosis was found in 31% of their population and increased up to 67% in obese subjects. Other studies^[36-38] have confirmed this positive correlation between BMI, waist-to-hip-ratio and hepatic steatosis. A few studies focusing on the natural history of NAFLD showed that only 1%-5% of patients with simple steatosis eventually develop actual cirrhosis^[39,40]. WHO estimated that at least 2 million people will develop cirrhosis due to hepatic steatosis. Taken all together, NAFLD has become the third most important indication for liver transplantation and will become the leading indication in the next decades. In this respect, the finding of NASH in young obese children is very alarming^[41]. Training in paediatrics, internal medicine, gastroenterology and hepatology should emphasize the awareness of this entity to avoid complications of portal hypertension, minimize the need for liver transplantation and prevent the associated cardiovascular disease.

This review will focus on the clinical characteristics, pathophysiology, imaging techniques and therapeutic opportunities of this disease.

CLINICAL CHARACTERISTICS

Most patients with NAFLD are asymptomatic and the symptoms are usually non specific when they occur. Frequent complaints are fatigue and vague right upper quadrant abdominal discomfort. Because of the latter, steatosis is often found at ultrasound examinations made for suspicion of biliary disease. Ultrasound abnormalities and elevated alanine transaminase (ALT) levels are often found at routine check-up or when patients present themselves with physical complaints due to other diseases. Given that NAFLD is widely accepted as a part of metabolic syndrome, or at least being related with it, most patients present with other pathologies linked to this syndrome. Once other pathologies (Table 1) are ruled out as a cause of steatosis, NAFLD can be allocated as the most common cause for elevated ALT levels and/or steatosis. Mildly raised levels of ALT have been found in hospitalized NASH patients, but not higher than four times the upper limit of normal (ULN)^[42-46]. These levels fluctuate but never return to normal values. Abnormal AST levels have also been found, especially in cirrhotic patients. Gamma-glutamyl transpeptidase (γGT) and alkaline phosphatase levels can increase although in unknown frequency. In

Table 1 Possible causes for steatosis hepatitis

Causes	
Metabolic	Abetalipoproteinemia Glycogen storage diseases Weber-Christian disease Wolmans disease Acute fatty liver of pregnancy Lipodystrophy Iron overload syndromes α-1-antitrypsin deficiency
Nutritional	Malnutrition Total parenteral nutrition Severe weight loss Refeeding syndrome Jejunio-ileal bypass Gastric bypass Jejunal diverticulosis with bacterial overgrowth
Inflammatory	HIV ¹ Chronic Hepatitis C infection
Drugs	Methotrexate Diltiazem HAART ² Amiodarone Glucocorticoids
Toxins	Alcohol Environmental hepatotoxins (e.g. toxic mushroom) Wilsons disease
Autoimmune	Autoimmune hepatitis Celiac disease

¹HIV: Human immunodeficiency virus; ²HAART: Highly active anti-retroviral therapy.

conclusion, these mild laboratory abnormalities would not be very helpful in diagnosing this disease due to their low sensitivity and specificity.

At more advanced disease stages, liver stigmata like jaundice, spider naevi and erythema palmare may develop. In these patients, laboratory abnormalities are consistent with progressed liver disease.

PATHOPHYSIOLOGY

Healthy subjects

Within the body's system, the liver plays a crucial role in controlling fatty-acid and triglyceride (TG) metabolism by synthesizing, storing, secreting and oxidizing free fatty acids (FFA). The liver responds to and manages fatty acids that originate from ingested foods, adipose stores and its own *de novo* production. Oxidation of FFA is considered the main energy source for gluconeogenesis in a fasting state. TG is incorporated into very low dense lipoprotein (VLDL) particles while being transported out of the liver to peripheral tissues. Fatty acids are mainly stored in adipose tissues of human beings. In healthy individuals, fasting lipolysis causes release of TG into the plasma nonesterified fatty acid (NEFA) pool, while adipocytes will take up fatty acids. Postprandial pancreas-released insulin

increases lipogenesis and decreases lipolysis and fatty acid oxidation in mitochondria. The second source of fatty acids contributing to the total liver supply is hepatic *de novo* lipogenesis (DNL). In healthy individuals, this source is a minor contributor while fasting and insulin levels are low. The third source of fatty acids is the absorption of dietary fats.

In 1998, Day *et al*^[47] launched the “two-hit-theory”, stating that two succeeding wallops have to be delivered to the liver to cause NASH. The first hit, development of hepatic steatosis, is the accumulation of TG consisting of 3 fatty acids and a glycerol backbone, in the hepatocytes. The development of hepatic steatosis is a form of ectopic lipid accumulation, resulting from a disturbance in the balance between supply, formation, consumption and hepatic oxidation or disposal of TG. Consumption includes mitochondrial β -oxidation, production of ketone bodies, and secretion of TG in VLDL particles. Many animal and human studies have shown that there is an inextricable relation between obesity and insulin resistance (IR)^[48-52]. IR is a key pathogenic factor for the development of hepatic steatosis^[26-34].

Insulin resistance

Insulin resistance (IR) is the disruption of signalling pathways in cells, leading to a diminished ability to execute normal cellular responses to insulin. For details of the insulin pathway, the reader is referred to excellent reviews by Herman *et al*^[53] and Taniguchi *et al*^[54]. In summary, the insulin receptor is tyrosine-phosphorylated upon binding to insulin, which in turn causes tyrosine phosphorylation of the insulin receptor substrate (IRS) proteins. There are two important IRS: IRS-1 and IRS-2. IRS-1 is the initiator in the pathway of glucose metabolism. Upon phosphorylation, IRS-1 induces stimulation of the phosphatidylinositol 3-kinase (PI3K)-AKT/protein kinase B (PKB) pathway, resulting in recruitment of glucose transporters (GLUT). IRS-2 cranks up lipid metabolism in cells and is a main regulator in DNL *via* sterol regulatory element binding protein 1c (SREBP-1c). SREBP-1c is a member of the SREBP family, a group of transcription factors that play a fundamental role in cellular lipid metabolism^[55]. Three different SREB proteins have been identified. These SREB proteins activate the complete program of cholesterol and fatty acid synthesis in the liver^[56]. SREBP-1c is the isoform that plays a role in synthesis of fatty acids and TG in the liver, by stimulating the formation of enzymes, most important acetyl CoA carboxylase (ACC) and fatty acid synthase (FAS)^[57]. Most SREBP-1c-stimulated enzymes are also regulated by carbohydrate response element binding protein (ChREBP)^[58]. For a long time, it was assumed that a defect in muscle tissue is the first step in the origination of insulin resistant states. In the last decade, however, this doubtful honour shifted towards the adipocyte. Besides the lump storage of fat, in the mid 90's, the exocrine functions of fat were recognised, and it became clear that fat is the choirmaster in the aetiology of IR^[59]. It has been found that mice lacking GLUT4 in adipocytes develop IR in muscle and liver tissue^[60], suggesting that fat cells secrete a substance that can induce IR in other tissues.

Adipocytes excrete a number of bioactive peptides that are collectively called “adipokines”. Leptin (Greek for ‘thin’), discovered by Zhang *et al* in 1994^[61] is the prototype, but since then other adipokines, such as adiponectin^[62], ghrelin^[63], resistin^[64] and recently retinol binding protein 4 (RBP4)^[65], have been identified and characterized. RBP4 works partly by blocking the action of insulin in muscle and liver^[65,66]. Depending on the amount of lipids, a stored adipocyte releases adipokines when its maximum storage is reached (leptin, RBP4) or more capacity available (adiponectin). Each adipocyte secretes a small amount of these peptides into its direct surroundings. In obese states, adipocytes also excrete inflammatory cytokines^[52]. It has been found that TNF- α is over expressed in obese people^[67]. Since this discovery, more inflammatory mediators have been recognised and investigated^[68]. Excretion of inflammatory cytokines attracts macrophages, probably as a natural response to the clearance of the extreme swollen body and malfunctioning of fat cells. Macrophages themselves also release inflammatory substances. In the copious blood flow in adipose tissue, these peptides readily manoeuvre into the blood, which enables them to exert a number of endocrine and autocrine functions. Together, all adipocytes make up the largest endocrine organ resulting in a considerable influence of adipokines on body function^[69-71]. Especially because adipocytes grow and proliferate in an overfed situation, this will lead to more excreted adipokines navigating lipids to certain specific areas of the body. Muscle and liver tissue are the main sites for ectopic fat accumulation^[72]. In myocytes and hepatocytes, FFA cause IR in genetically susceptible subjects through defects in the insulin signalling pathway. Although the search for specific defects in the pathogenesis is complicated by the complexity of insulin signalling cascades, one of the major problems is a disturbance in the IRS1/PI3-kinase/Akt/GLUT pathway. IRS-1 tyrosine phosphorylation leads to serine phosphorylation, thereby interrupting the pathway for the transport of glucose via the GLUT transporters to the membrane. A number of inflammatory kinases have been found to induce this inhibitory serine phosphorylation, such as I κ B-kinase- β (IKK- β), jun-kinase-1 (JNK-1) and suppressor-of-cytokine-signalling-3 (SOCS3). Interestingly, SOCS3 receptors have also been found in the hypothalamus, where it may be involved in leptin signalling. JNK-1 is found to be an important mediator in the development of inflammation in obese tissue^[73]. Özcan *et al*^[74] found that the protein is triggered by endoplasmatic reticulum (ER) stress. In obese states, the metabolic demand on the ER, is maximal and sometimes even more. As a response to the continuously high workload, the ER initiates a complex response system, referred to as the unfolding protein response (UPR). This UPR leads to the activation of JNK-1, IKK- β and TNF- α . More evidence comes available that the ER in this way translates the metabolic stress into an inflammatory signal.

Interestingly, it has been found that in hepatic IR states, the IRS-2 signalling is relatively intact, insulin down-regulates the IRS-2 receptor, resulting in over-expression of SREBP-1c and up-regulation of DNL^[75].

Supply

Plasma FFA: The plasma nonesterified fatty acid (NEFA) pool contributes to the majority of fatty acids that flow to the liver in the fasting state, thus providing the bulk of FFA secreted by the liver in VLDL particles^[76]. The storage of TG and FFA in adipose tissue is mediated by insulin, especially in visceral fat. In healthy individuals, consumption of a meal induces an increase in plasma insulin concentration and subsequent suppression of adipocyte lipolysis, thereby reducing the plasma NEFA pool. Adipose tissues, especially visceral adipocytes, function as a depot for energy that can be released in times of need. IR develops after long-term excess energy intake, thus decreasing the inhibitory effects of insulin on peripheral lipolysis and increasing the availability of FFA. FFA is released into the blood stream by flow of visceral adipocytes to the liver without any circumbendibus. Paradoxically, the contribution of FFA derived from the plasma pool flowing into the liver is relatively smaller in NAFLD patients compared to healthy subjects. This is due to the increased contribution of other mechanisms in these patients.

Dietary fat intake: Dietary fats are supplied to the liver by two different routes. Chronic intake of energy-enriched food challenges the processing capacity of adipocytes, with an overflow of NEFA into the plasma as a result^[77]. Lipid accumulation in non-adipose tissue, mainly muscle and the liver, is a characteristic of obesity, but is also seen in lipodystrophy. Where in obesity the adipocytes overflow, in lipodystrophy there are no or insufficient adipocytes to store lipids, both being a factor for the increased ectopic lipid accumulation. A second route is *via* remnant chylomicrons. FFA and monoglycerides are absorbed separately and packaged into TG in intestinal epithelial cells. They are then secreted in chylomicrons (lipoproteins with a very high lipid content), which release FFA to adipose and muscle cells, mediated by lipoprotein lipase. Chylomicrons depleted of most lipids (known as chylomicron remnants) are absorbed by the liver. Studies have shown that in the remnant delivered to the liver, up to 50% of the FFA can still be present, which then have to be processed by the liver. Dietary fat intake is responsible for approximately 15% of the FFA supply to the liver^[78].

De novo lipogenesis (DNL): The term lipogenesis refers to the biosynthesis of lipids. DNL indicates that synthesis of fatty acids occurs in various non-fat precursors. Most important precursors are glucose, amino acids and ethanol which produce acetyl-CoA during their catabolism and are therefore susceptible to conversion to fatty acids in the intermediary metabolism. SREBP-1c plays a key role in the regulation of DNL and is activated by insulin, endocannabinoid receptor CB-1^[79], liver X receptor (LXR)- α ^[80], oxysterol binding protein^[81] and suppressor of cytokine signalling (SOCS)-3^[82]. Leptin and glucagon have antagonising effects. The suppressing effect of leptin on SREBP-1c seems paradoxical, as obese persons often exhibit high levels of leptin and high expression of SREBP-1c. This is the consequence of, on the one hand, an increase in leptin production by the expanding mass of fat cells, and on the

other hand, a decrease in leptin sensitivity^[83].

LXR- α is an oxysterol-activated nuclear receptor. Activation of LXR- α induces SREBP-1c transcription through the co-activation of retinoid X receptor (RXR)- α . Grefhorst *et al.*^[84] showed that exogenous administration of LXR- α ligands results in extensive hepatic steatosis.

The observation of an increase in appetite in association with the use of cannabis has led to the hypothesis that the endocannabinoid pathway might play a role in energy intake and fat metabolism. The most prominent receptors are the cannabinoid receptor 1 (CB-1) and cannabinoid receptor 2 (CB-2). CB-2 is mostly expressed in the immune system, while CB-1 is found to be involved in the SREBP-1c pathway in liver and brain. In these pathways, the effect of CB-1 is twofold that of CB-2. Regulation of the hypothalamic-driven feeding behaviour^[85,86], has direct effects on energy intake. A second effect is on hepatic fatty acid synthesis, hepatic TG quantity and activation of the released fatty acids from adipose tissue^[79,87].

SOCS3 is an adipocyte-excreted cytokine that up-regulates hepatic SREBP-1c. Although the mechanisms have not been fully elucidated, TNF- α , interleukin (IL)-6 and leptin seem to augment excretion of SOCS3, whereas adiponectin is found to have inhibitory effects^[82]. In healthy individuals, DNL is a minor supplier of fatty acids to hepatocytes in the fasting state, when insulin levels are low. Less than 5% of the total supply of fatty acids originates from DNL. In the postprandial state, insulin stimulates DNL which then accounts for over 26% of the FFA supplies. This more or less diurnal rhythm is not seen in NAFLD patients where the contribution of DNL is continuously 26%^[78].

Oxidation

In normal conditions, mitochondria take up FFA as a substrate for β -oxidation while fasting fatty acid oxidation is the main substrate for the production of energy used in gluconeogenesis. In IR states, the amount of FFA available for oxidation exceeds the mitochondrial capacity. A bulk of acetyl CoA enters the citric acid cycle, resulting in the delivery of electrons to the respiratory chain, where they generate reactive oxygen species (ROS).

Outflow

In physiological conditions, transport of TG from hepatocytes occurs through formation of VLDL by the ER in two steps. The first step is the lipidation of apolipoprotein B (ApoB), which creates a so-called pre-VLDL. This lipidation of ApoB is catalyzed by the microsomal triglyceride transfer protein (MTTP). The pre-VLDL is transported to the smooth ER and further lipidated before its migration to the cell membrane again. MTTP is the catalyst in this process. The second step is progression of ApoB to pre-VLDL, which is dependent on the amount of TG available. If insufficient lipids are available, the ApoB protein will degrade. Insulin is a strong promoter of ApoB degradation *via* the PI3K pathway and can thus influence the number of VLDL particles synthesized. SREBP-1c inhibits the formation of MTTP, thereby reducing the amount of VLDL particles produced. In IR

states, the PI3K pathway is eliminated to a certain extent but the up-regulated SREBP-1c leads to a decrease in VLDL synthesis. The size of the particle is dependent on the amount of TG stored in cells. It has been shown that VLDL particles in fatty livers are sufficiently larger, most likely as a result of the decreased production^[84]. It is likely that export of TG is impaired in IR states.

From hepatic steatosis to NASH

Steatohepatitis is characterized microscopically by hepatic fat accumulation, mixed lobular inflammation, ballooning degeneration of hepatocytes, Mallory bodies, glycogenated hepatocyte nuclei, and pericellular fibrosis. The characteristic “chicken wire” pattern of pericellular fibrosis affects portal areas only at later stages. Accumulation of FFA and TG in hepatocytes by mechanisms described above spreads over the bed for the second hit in Day’s widely accepted theory^[42]. By far, not all fatty livers progress to steatohepatitis^[39,88]. Although a considerable amount of evidence is available for environmental influences, there is inevitably a genetic compound that contributes to the origination and progression of the disease. Factors responsible for the progression from simple fatty liver to NASH are extensively researched. TNF- α expression, lipid peroxidation and mitochondrial dysfunction are likely to be involved. The mechanism is triggered and starts a sequence that leads to inflammatory response and release of inflammatory cytokines, eventually resulting in the development of fibrosis and cirrhosis. Since the lipid-laden liver of steatotic patients does not increase in size, the entire lipid content must come in place of existing structures and compress them somewhat. To resolve this disadvantageous condition, hepatocytes need to clear FFA and TG, for which they can use two mechanisms. The first is the above described excretion of TG through formation of VLDL particles. It is postulated that this mechanism is impaired in hepatic steatosis. The other mechanism is the metabolism of FFA by mitochondrial β -oxidation^[89]. In summary, these metabolic changes in steatotic livers result in the formation of reactive oxygen species (ROS) by mitochondria as a result of increased mitochondrial β -oxidation, hepatic microsomal cytochrome P450 2E1 (CYP2E1) up-regulation and formation of Kupffer cell ROS.

The mechanism by which mitochondrial β -oxidation is up-regulated in steatotic livers still remains unclear, but it is thought that especially DNL-derived FFA and peroxisome proliferator-activated receptor (PPAR)- α are important stimulators of carnitine palmitoyltransferase-1 (CPT-1) responsible for the entry of FFA into mitochondria. The massive influx of FFA from peripheral tissue and mostly the increased DNL within hepatocytes exceeds the metabolic capabilities of mitochondria, with the formation of ROS and an inflammatory response as a result.

CYP2E1 is predominantly found in the ER, but significant amounts are present in the cytosol and mitochondria where it stimulates microsomal fatty acid oxidation. Increased CYP2E1 activity and expression are found in NASH patients^[90], but the mechanisms behind this remain unclear. Recently Rahman *et al* found that CCAAT/Enhancer binding protein (C/EBP β) expression may be

an important factor in the upregulation of CYP2E1^[91]. Other authors have postulated the influence of IR^[92,93] and increased ketogenesis^[94].

In various models, steatosis endotoxin receptors on Kupffer cells are increased, which may trigger the assemblage of NAD(P)H oxidase on the plasma membrane of hepatocytes and thereby causing ROS formation^[95].

The surplus of FFA within the liver causes the formation of excess amounts of ROS. Mitochondria in non-fat-laden hepatocytes also produce rather large amounts of ROS, but enzymatic processes can change ROS into “safe water”. Excessive ROS formation can lead to an overburden of this escape mechanism and ROS can leave the mitochondria. In the cytosol, ROS enhances lipid peroxidation products and mitochondrial DNA (mtDNA). Increased mitochondrial ROS formation could also directly oxidize mitochondrial DNA, proteins and lipids, and trigger hepatic TNF- α formation by activating nuclear factor (NF)- κ B and deplete antioxidants, thus further increasing mitochondrial ROS formation. Induction of the inflammatory cascade can also be the result of the reduced availability of anti-inflammatory products as adiponectin^[96].

Another worsening and possible triggering factor might be the tumour necrosis factor (TNF)- α ^[97,98]. In obese and IR patients, serum levels of TNF- α are proportionally increased and one of the striking differences between patients with NASH and those with simple steatosis is the serum level of TNF- α ^[99]. Not hepatic, but mainly adipose tissue is the main supplier of TNF- α . In normal conditions, hepatocytes that frequently come into contact with a variety of endo- and exotoxins are not very sensitive to TNF- α , but in NASH patients it might be possible that the increased levels cause leakage of the mitochondrial outer membrane and thereby increasing ROS formation^[89]. TNF- α also has direct effects on IRS phosphorylation and stimulates SOCS3 formation *via* interleukin 6 (IL-6) and inhibitory κ B-kinase (IKK), thereby worsening IR and steatosis^[100,101] (Figure 1).

DIAGNOSIS

General

As noted before, NAFLD patients are mostly asymptomatic because slightly elevated liver enzymes are accidentally found. There is some debate about the question when further investigations are performed, especially in liver biopsy. Generally, it is reasonable to undertake action when the ALT level is $> 2 \times$ ULN measured at two different occasions^[102]. When the diagnosis of NAFLD is considered, it is important to exclude other pathological conditions that are associated with elevated ALT levels and/or steatosis (Table 1). It is especially difficult to find the difference between alcoholic and non-alcoholic liver diseases, as not all patients are honest about their alcohol intake and there is no adequate diagnostic difference between the two diseases. An abdominal ultrasound is performed to exclude hepatobiliary obstructions or tumours.

Imaging techniques

Ultrasonography, computerized tomography (CT) scan,

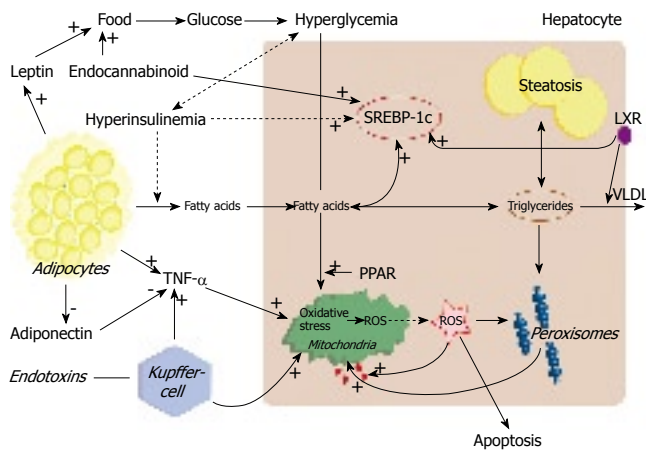


Figure 1 Pathogenesis of nonalcoholic steatohepatitis during insulin resistance. FFA is supplied to the liver through dietary intake, and lipolysis in adipocytes via chylomicron remnants. Transcription of SREBP-1c is chronically up-regulated resulting in DNL. Simultaneous inhibition of VLDL synthesis results in disruption of triglycerides export. The surplus of fatty acids is stored in triglycerides or metabolized via peroxisomal and mitochondrial oxidation. The excessive oxidation will lead to production of ROS and oxidative stress. This will trigger the inflammatory response and apoptosis as well activation of stellate cells.

and magnetic resonance imaging (MRI) can all be used to diagnose hepatic steatosis. Ultrasonography, the most commonly used and least expensive method, can be used to diagnose moderate to severe steatosis. Studies in the 1980's found that its sensitivity and specificity vary from 60% to 94% and 88% to 95%, respectively. Palmentieri *et al.*^[103] investigated a subgroup of patients with hepatic steatosis > 30%, showing that the sensitivity and specificity of US increase up to 90% and 97%, respectively. Although the diagnostic capacity of ultrasonography increases with higher degrees of steatosis^[103-105], accurate quantification of hepatic fat and comparison between simple hepatic steatosis and steatohepatitis are impossible. Un-enhanced CT imaging can accurately detect and quantify the amount of steatosis in patients^[106,107]. Grey scales (representing the amount of radiation absorbed) of the liver and spleen are measured and expressed in Hounsfield units (HU). For quantification of the amount of fat-infiltrated hepatocytes, the difference in grey scale between the liver and spleen can be measured in HU. This measurement correlates well with the percentage of hepatocytes with fatty infiltration^[107], where the hepatic attenuation decreases with the increased fat. For steatosis > 33%, the sensitivity and specificity are 82%-93% and 100%, respectively^[106]. There is no difference in diagnostic value between a non-contrast CT scan and a contrast-enhanced one, with specifically more attenuation in the blood vessels than in the liver parenchyma. Contrast-enhanced CT scan does not provide more information. In fact, its sensitivity and specificity for steatosis are lower than those of un-enhanced CT imaging. Another drawback of contrast-enhanced CT scan of steatosis is the greater difficulty in its measurement due to the more complicated protocol.

The most accurate available technique for detection and quantification of hepatic steatosis is NMR^[108-110]. T1-weighted dual-echo chemical shift gradient-echo NMR is commonly used to obtain images. The main advantage of

this technique is the possibility of acquiring in-phase (water) and opposed-phase (fat) images in one breath hold, thereby reducing the influence of breathing movements and contrast absorption. Besides, this technique is useful for follow-up due to the lack of the use of fluoroscopy. Qualitative measurement or detection of steatosis is assessed on opposed-phase images. On T1-weighted images, a shorter relaxation time represents the higher signal intensity (SI). In healthy individuals, the SI of the liver is higher than that of the spleen. Steatosis causes a drop in SI on opposed-phase images. When SI of the liver equals to that of the spleen, a diagnosis of mild steatosis is made. Moderate or severe steatosis is diagnosed when SI of the liver is less than that of the spleen. Quantification of steatosis is possible with MRI by calculating the mean SI of the liver on in-phase and opposed-phase images. A number of regions of interest (ROI) are drawn in sections of the liver, from which the mean can be calculated. Using the same method, the mean SI of the spleen or fat issue surrounding the liver can be calculated as a reference. The difference in SI on opposed-phase and in-phase images can be calculated and expressed as a percentage of fatty infiltration of the liver. MRI shows a good correlation with histological examination, the sensitivity and specificity of MRI are 100% and 92.3%, respectively^[110].

Another relatively new NMR technique under development, widely used to quantify hepatic triglyceride content, is magnetic resonance spectroscopy (MRS)^[111,112]. MRS of *in vivo* biological tissues was first reported in 1973 and used in the field of chemistry before NMR was introduced in hospitals. The principle of MRS is based on the differences in resonance frequencies of protons. The electron cloud surrounding molecules shields protons to varying degrees depending on the specific molecule structure and the specific position of protons in the molecule. This shielding causes protons in different molecules or even in different places of the same molecule to have a slightly different resonance frequency. Instead of using resonance frequencies for creating anatomical images, the differences in spectroscopy frequency are used to identify different chemical compounds. By using these differences, protons in water molecules can be differentiated from protons in lipids. Quantifying the amount of a certain biochemical component is possible by calculating the area under the "fat resonance peak" and comparing it to the "water resonance peak" (Figure 2).

Technically, coronal, axial and sagittal images of the liver are acquired and a volume of interest is defined, avoiding major blood vessels and bile ducts.

Unfortunately, to date, no conventional diagnostic imaging method that can accurately distinguish NASH from simple steatosis is available^[111,113]. Transient elastography (fibroscan) is a technique under development. Studies in chronic hepatitis C (CHC) patients showed that cirrhosis (severe fibrosis) can adequately be distinguished from mild fibrosis, but this accuracy is much less in distinguishing various degrees of fibrosis^[114]. Another drawback of the fibroscan method is that it is difficult to use and inaccurate in obese patients. A combination of serologic markers is under investigation to assess the severity of fibrosis^[115]. Although results seem promising, further study is wanted.

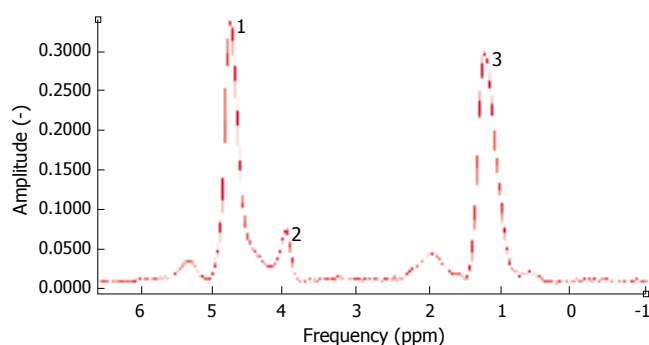


Figure 2 Spectrum of a fatty liver measured by ^1H -magnetic resonance spectroscopy. The water peak is at 4.3 ppm. 1: Residual water partially suppressed; 2: Glycerol/phospholipids; 3: $(-\text{CH}_2-)_n$ of saturated fat.

THERAPEUTIC OPTIONS

Life style modifications and weight reduction

Since the majority of patients suffer from obesity, insulin resistance and concomitant cardiovascular disease, weight reduction of approximately 10% has been advised by the American Gastroenterological Association^[116]. An analysis by Wang *et al*^[117] of all published articles and meeting abstracts have revealed no randomized controlled trials. Besides, the use of variable primary endpoints and control groups worsened the analysis of this comprehensive review. Although on a theoretical basis, reduced caloric intake, exercise and weight loss would eventually improve hepatic steatosis, very scarce evidence is available to support this hypothesis. The limited data are due to the small number and lack of histological evidence. Recently, Huang *et al*^[118] analyzed the effect of a 12-mo standardized nutritional counseling in 16 of 23 patients and found that the mean weight reduction is 2.9 kg with histological improvement in 9 patients.

No data are available on the long-term effect of weight loss on liver-related diseases such as cirrhosis or its complications.

Pharmacological interventions

Drug-induced weight reduction: The only two registered drugs for pharmacological weight reduction, Orlistat ($n = 4$) and Sibutramine ($n = 1$), have been investigated in a few small non-randomized studies^[119-123]. Orlistat, a gastric and pancreatic lipase inhibitor resulting in fat malabsorption (approximately 30%), has been studied in one case series^[119], three pilot studies^[120,121,123] and one RCT^[124]. Overall, patients can achieve impressive weight loss (10-15 kg) with improvements in liver enzymes but variable results in histology.

Sibutramine, a serotonin and norepinephrine reuptake inhibitor, acts on enhancing satiety *via* central mechanisms. There is only one published study on NAFLD in 25 patients demonstrating weight loss, improvement in liver enzymes and hepatic steatosis on ultrasound. Unfortunately, repeated liver biopsy was not performed.

Antioxidants: Since the pathogenesis of NAFLD is thought to be in a two-hit fashion, it is believed that oxidative stress

causes a second hit leading to inflammation. *In vitro* and *in vivo* animal and human studies^[125,126] have been performed on the effects of vitamins E and C as antioxidants. The promising results in one study were counteracted by another. Harisson *et al*^[126] reported that a between group analysis cannot show any beneficial effect of the combination of vitamins E and C after 6 mo, compared to placebo. Recently, Lirussi *et al*^[127] identified 6 trials that were analyzed according to the intention-to-treat principle and found that despite the significant improvements in liver enzymes and minor adverse events, radiological and histological data are too limited to support or repudiate the use of antioxidants in patients with NAFLD.

Ursodeoxycholic acid (UDCA): UDCA, approved as a drug of choice in treatment of patients with PBC, exerts its effect by reducing the portion of hydrophobic bile acids contributing to oxidative stress. Four clinical trials^[128-131], of which only one assessed histology^[129] and had a low-bias risk, have been conducted. No significant differences in the degree of steatosis, inflammation or fibrosis could be found between the treated and placebo groups. Unfortunately, these studies had no heterogeneity with respect to inclusion criteria, sample size, duration of treatment and methods of outcome assessment. Therefore, the Cochrane analysis by Orlando *et al*^[132] concludes that the data are insufficient to use UDCA in treatment of patients with NAFLD.

Metformin: Metformin, a biguanide, has been shown to be an effective drug for the treatment of patients with type 2 diabetes mellitus^[133]. Its administration improves hepatic steatosis and hepatomegaly. In addition to this observation, human pilot studies performed with variable results^[134,135] could not demonstrate a beneficial effect of metformin compared to a calorie-restricted diet. Similar to the previous mentioned therapies, histological data are limited to support an association between improvements in liver enzymes and histological findings.

Thiazolidinediones (TZD): This class of agents acts as agonists of the peroxisome proliferator-activated receptor gamma on ameliorating insulin resistance and glucose and lipid metabolism. The first generation of TZD, e.g. troglitazone, appears to be effective on ALT levels, although it has been withdrawn from the market due to its severe hepatotoxicity^[136,137]. In addition, the second generation of TZD (pioglitazone and rosiglitazone) is safer. A Cochrane analysis by Angelico *et al*^[138], (excluding trials treating patients with type 2 diabetes mellitus) extracted only one RCT by Sanyal *et al*^[139], showing that combination therapy with vitamin E and pioglitazone is significantly superior to vitamin E alone in terms of the degree of hepatic steatosis, but not other histological variables.

An open label trial of rosiglitazone in 26 biopsy-proven NASH^[140] and two pilot studies with pioglitazone^[141,142] ($n = 73$) during 48 wk demonstrated improvements both in liver chemistry and in histological features like steatosis, necroinflammation and fibrosis. Weight gain seems to be the most important adverse event. Recently, the use of rosiglitazone has been associated with the slightly

increased bone loss in postmenopausal women and elderly diabetics^[143-145] and an increased risk of cardiovascular events, i.e. myocardial infarction^[143,146,147]. Further research focusing on larger randomized controlled trials with this class of drugs will be valuable.

Lipid lowering drugs: Use of statins or fibrates has not been investigated in large randomized trials. Primary endpoints of these studies are liver enzymes but not histology. No definite conclusions can be made from these limited studies^[121,148].

Adipokines: As mentioned before, adipocytes act as hormonal active tissue producing cytokines, like leptin, resistin, adiponectin and tumor necrosis factor- α (TNF- α). It was reported that levels of TNF- α are increased in patients with NASH^[99]. TNF- α , as a potential proinflammatory cytokine, promotes insulin resistance and thereby hepatic steatosis^[101]. Some animal and even fewer human studies focusing on the effect of blocking this adipocytokine showed that patients treated with pentoxifyllin for 6 or 12 mo have a significant improvement in liver enzymes^[149,150].

Administration of synthetic adiponectin, exposing opposite effects to TNF- α , in two animal models can ameliorate hepatomegaly, steatosis and elevated ALT-levels^[151].

Leptin, a 16-kDa protein hormone, has been shown to play a pivotal role in energy homeostasis by activating and inhibiting certain neurons^[152]. It has been shown that administration of leptin infusions in patients with generalized lipodystrophy significantly ameliorates insulin resistance, glucose and triglyceride levels as well as hepatic steatosis^[153,154]. No such studies have been performed yet in NAFLD patients.

Resistin, another adipocytokine, is a subject of controversy regarding to its causal role in obesity and type 2 diabetes mellitus. Due to this controversy, no published data are available about its beneficial effects on inhibition of resistin.

FUTURE DIRECTIONS

Up until now, the exact treatment strategy for the treatment of patients with NAFLD has not been well established in RCT. Research topics in this field are challenging. In the United States and Europe, some research groups are focusing on comparing different treatment options and identifying those patients most in need for treatment. Evolving new imaging techniques like proton magnetic resonance spectroscopy might differentiate between those patients having type 1 NAFLD (defined as simple steatosis without features of inflammation) and type 2 NAFLD (containing patients with variable grades of inflammation and fibrosis eventually resulting in cirrhosis).

Several registered drugs aiming at improving metabolic syndrome should be further investigated on their exact anti-steatotic effects. The most promising drugs are insulin sensitizers (especially Thiazolidinediones), which should be further investigated in a larger RCT aiming at establishing

the optimal dosage, time of treatment and adverse effects.

Since the discovery of two cannabinoid receptor antagonist receptors (CB1 and CB2) in the late 1980's and the beginning of the 1990's in the brain^[155,156] and gastrointestinal tract^[157], it has gained more and more interests from both researchers and clinicians. CB1 has been extensively found in the central nervous system, affecting many neurological and psychological phenomena, like appetite, mood and spatial coordination of muscle tone^[158]. In contrast, CB2 detected in peripheral cells of the immune system (lymphocytes, monocytes and neutrophils) exerts additional effects on the gut (inhibition of motility) and vasodilation^[159,160]. Research in animal models revealed that activation of the endocannabinoid system leads (partially) to the development of portal hypertension and arterial hypotension through macrophages and platelets activated by bacterial lipopolysaccharides^[161,162]. Secondly, anandamide, an endogenous ligand for CB-receptors, appears to be up-regulated in patients with endotoxic shock^[163] and subsequently hepatocellular apoptosis has been linked to anandamide and its lipid-lipid plasma membrane interaction, resulting in enhanced susceptibility to oxidative stress^[164]. Thirdly, another hypothesis is that an activated CB-system may have influence on hepatic encephalopathy^[165]. In humans, endocannabinoids have been used in the treatment of three patients with cholestatic-related intractable pruritus^[166]. SR141716A (Rimonabant, Sanofi-Aventis, Paris, France) under investigation may be used in the treatment of patients with obesity and metabolic syndrome^[167]. In obese Zucker rats, administration of this drug could ameliorate markers of hepatic damage (defined as increased liver enzymes, focal hepatic TNF- α and decreased adiponectin), and decrease hepatomegaly^[167]. Preliminary results in humans are promising since the used drug is safe, effective in achieving weight reduction and amelioration of the lipid profile and metabolic syndrome^[85,168,169].

In conclusion, the development of drugs acting both on the cannabinoid system influencing the central nervous system through inhibition of appetite and on the peripheral tissue ameliorating hepatic hemodynamics, inflammation and weight reduction accompanying improvement in metabolic syndrome, will lead to new research aims in the field of hepatology. In particular, the development and conductance of RCT with synthetic, non-psychotropic cannabinoids might result in optimizing treatment strategies for patients with NASH.

CONCLUSION

Nonalcoholic fatty liver disease, especially nonalcoholic steatohepatitis, forms a definite threat to human health. With the increase in obesity, an increase in NAFLD patients can be expected, eventually leading to an increased number of liver transplantations. Pathophysiological mechanism is the subject of research all around the world, leading to a continuous current of new evidence and more knowledge about the complex mechanisms behind the disease. At the same time, diagnostic methods for detecting steatosis and steatohepatitis are under development.

¹H-magnetic resonance spectroscopy is accurate in the detection and quantification of fat in the liver, but the eagerly wanted non-invasive tool for the detection of fibrosis or inflammation in steatotic livers is not available. Therapeutic options do increase. Weight loss by dietary and lifestyle intervention remains the cornerstone of treatment, but motivated patients can be supported by medications. Most promising results are found with thiazolidinediones, but recent upheaval around rosiglitazone means a setback of this drug.

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Effect and mechanism of the *Twist* gene on invasion and metastasis of gastric carcinoma cells

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Abstract

AIM: To study the effect of the transfected *Twist* gene on invasion and metastasis of gastric carcinoma cells and the possible mechanisms involved.

METHODS: Human gastric carcinoma MKN28 cells were stably transfected with *Twist* sense plasmid, and MKN45 cells were stably transfected with *Twist* antisense plasmid using the lipofectamine transfection technique. RT-PCR, Western blotting, EMSA, gelatin zymography assay, and *in vitro* invasion and migration assays were performed. Nude mice metastasis models were established by the abdominal cavity transfer method.

RESULTS: Cell models (*TwistS*-MKN28) that steadily expressed high *Twist* protein were obtained. Compared with MKN28 and pcDNA3-MKN28 cells, adherence, migration and invasion ability of *TwistS*-MKN28 cells were clearly raised. The number of cancer nodules was increased significantly in the abdominal cavity and liver of nude mice inoculated with *TwistS*-MKN28 cells. Overexpression of *Twist* in MKN28 cells increased Tcf-4/Lef DNA binding activity, and promoted expression of Tcf-4's downstream target genes cyclin D1 and MMP-2. However, suppression of *Twist* (*TwistAS*-MKN45) inhibited MKN45 cell invasion and the expression of cyclin D1 was reduced. The activity of MMP-2 was also decreased.

CONCLUSION: These results indicate that *Twist* promotes gastric cancer cell migration, invasion and metastasis, and *Twist* may play an important role in Wnt/Tcf-4 signaling.

INTRODUCTION

Gastric cancer is one of the most common cancers in the world. Several lines of evidence implicate the Wnt signaling pathway as a contributor to gastric carcinogenesis^[1]. In the presence of certain Wnt proteins, or due to the loss of tumor suppressors such as *APC*, glycogen synthase kinase-3 (GSK-3) activity is inhibited, which results in inhibition of β -catenin phosphorylation and inhibition of degradation. The resulting accumulation of β -catenin leads to the activation of the Tcf-4/Lef transcription factor, which up-regulates the expression of downstream target genes. People with a germ-line mutation of the *APC* tumor suppressor gene have a 10-fold increased risk of developing gastric cancer as compared with normal individuals^[2]. Mutations in the *APC* gene have been found in sporadic gastric cancer^[3]. β -catenin mutations have also been detected in intestinal-type gastric carcinoma tissues and gastric cancer cell lines^[4]. Based on these studies, we conclude that the Wnt/Tcf-4 signaling pathway is very important in gastric cancer cells.

Besides transforming growth factor β and receptor tyrosine kinase/Ras signaling, autocrine factors and Wnt-dependent pathways are reported to contribute to epithelial mesenchymal transition (EMT). EMT is a process whereby epithelial cells lose polarity and cell-to-cell adhesion, and undergo dramatic remodeling of the cytoskeleton. Concurrent with loss of epithelial cell adhesion and cytoskeletal components, cells undergoing EMT acquire

expression of mesenchymal components and a migratory phenotype. EMT was first recognized in embryogenesis in the early 1980s. Today, evidence is growing that carcinoma cells activate the dormant EMT program in promoting cell migration, invasion and metastasis^[5-7]. However, its pathogenesis in human carcinoma is obscure.

Several key inducers of EMT are transcription factors that repress E-cadherin expression, such as Snail, Slug, SIP1 and *Twist*. Recent studies have shown that *Twist*, a highly conserved basic helix-loop-helix protein that is essential for early embryogenesis, promotes EMT and plays an essential role in metastasis in a breast tumor model^[8]. *Twist* has also been suggested to have oncogenic properties. Overexpression of *Twist* in rhabdomyosarcoma inhibits myc-induced apoptosis and interferes with p53 tumor suppression^[9]. Up-regulation of *Twist* is associated with malignant transformation in T-cell lymphoma^[10]. Forced expression of *Twist* triggers resistance of human cancer cells to drugs that inhibit microtubule formation, such as taxol and vincristine^[11]. Furthermore, expression of *Twist* has been implicated in promotion of metastasis and invasive pathological subtypes in several types of carcinoma^[12,13].

However, the effect and mechanism of *Twist* gene on invasion and metastasis of gastric carcinoma remain enigmatic. Therefore, in the present work, two gastric cancer cell lines with different differentiation were steadily transfected with sense and antisense *Twist* vectors. The effect of *Twist* gene on cell migration, invasion and metastasis was investigated.

MATERIALS AND METHODS

Cell culture

Human gastric carcinoma cell line MKN28 was kindly provided by Dr. JI Shuyu (Kunming Medical college, Kunming, China). Gastric carcinoma cell line MKN45 was purchased from Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). Cells were cultured in RPMI 1640 medium (Gibco Biocult, Paisley, UK) that contained 10% fetal bovine serum at 37°C in a humidified 5% CO₂ atmosphere.

Plasmids and reagents

The *Twist* sense and antisense expression vectors (pcDNA3/*TwistS*, pcDNA3/*TwistAS*) were kindly provided by Dr. Glackin C^[14]. The identity of *Twist* was confirmed by gene sequencing and routine agarose gel electrophoresis. Primary antibodies (anti-*Twist*, anti-MMP-2 and anti-β-actin) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and secondary antibody (HRP-linked IgG) was from Cell Signaling Technology (Beverly, MA, USA).

Generation of stable transfectants

Cell transfection was carried out using Lipofectamine 2000 according to the manufacturer's instructions. Briefly, cells were grown to 80%-90% confluence, without antibiotics. Vectors that contained the different constructs (10 μg) were diluted in RPMI 1640 (100 μL) and mixed with the transfection solution for 15 min. After washing, the cells

were incubated with the transfection mixture at 37°C for 10 h, and then allowed to grow in fresh medium. Stable transfectants were isolated by selection with 600 mg/mL G418 (Geneticin; Amresco, Solon, OH, USA) for 2 wk. Pools of geneticin-resistant clones were passaged and expanded for Western blot analysis. Cells transfected with the pcDNA3 vector were used as controls.

Western blotting

Cell total proteins were prepared in SDS sample buffer and boiled for 3 min. Equal amounts of cell protein, quantified by the BCA Protein Assay kit (Pierce Biotechnology, Rockford, IL, USA), were loaded onto 10% SDS-PAGE. After electrophoresis, the separated proteins were transferred to nitrocellulose membranes. The membranes were stained with ponceau (Amresco) and blocked with 5% non-fat milk for 1.5 h, and then incubated with antibody for 18 h at room temperature. The blots were subsequently incubated with an HRP-conjugated secondary antibody. Proteins were visualized using 3,3'-diaminobenzidine, with β-actin as a control.

Extraction of nuclear protein

Cells were lysed in 400 μL ice-cold buffer A (10 mmol/L HEPES, pH 7.9, 10 mmol/L KCl, 0.1 mmol/L EDTA, 0.1 mmol/L EGTA, 1 mmol/L DTT, 0.5 mmol/L PMSF) by gentle pipetting. The cells were allowed to swell on ice for 15 min, then 40 μL of a 10% solution of Nonidet P-40 was added, and the tube was vigorously vortexed for 10 s. The homogenate was centrifuged for 30 s in a microfuge. The supernatant was transferred and the nuclear pellet was lysed with 50 μL buffer C (20 mmol/L HEPES, pH 7.9, 0.42 mol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 1 mmol/L DTT, 1 mmol/L PMSF), and the tube was vigorously rocked at 4°C for 15 min on a shaking platform. The nuclear extract was centrifuged for 5 min in a microfuge at 4°C. The supernatant containing nuclear protein was quantified by the BCA Protein Assay kit (Pierce Biotechnology) and stored at -70°C.

Electrophoretic mobility shift assay (EMSA) of TCF-4 DNA binding activity

EMSA was performed with the LightShift Chemiluminescent kit (Pierce Biotechnology). Specific oligonucleotides for binding of TCF-4 (S: 5'-CCCTTGTGATCTTACC-3'; A: 3'-GGTAAGATCAAAGGG-5') were prepared by end labeling of the 5' terminus with biotin (synthesized by Bioasia Biotech, Shanghai, China). Briefly, nuclear extract protein (10 μg) was incubated with reaction mixture [10 × binding buffer, 1 μg/L poly (dI.dC), 1% NP-40, labeled probe] for 20 min at room temperature. Each sample was electrophoresed in 6% non-denaturing polyacrylamide gel at 100 V for 1.5 h. The gel was then electrophoretically transferred to a nylon membrane. Finally, the membrane was detected by chemiluminescence. In competitive studies, a 100-fold excess of unlabeled probe was included in the reaction mixture.

RT-PCR

Cells were lysed in TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and total RNA was prepared according to

the manufacturer's instructions. cDNA was synthesized by the SuperscriptTM First Strand Synthesis System (Life Technologies, Gaithersburg, MD, USA). The cDNA was then amplified by PCR with specific primers, cyclone D1 sense: 5'-ACGGCCGAG-AAGCTGTGCAT; antisense: 5'-TTCCAATCCGCCCTCCATGGA. The cDNA of GAPDH was amplified as a control for the amount of cDNA present in each sample (sense: 5'-ACGGATTTGGTCTGATTGGG antisense: 5'-TGATTTTGGAGGG-ATCTCGC). The relative expression levels were generated by comparing the density to the controls and indicated underneath each gel.

Evaluation of cell adherence

Fifty milligrams per liter Matrigel solution diluted with sterilized double distilled water (1:8) was prepared, added to 96-well plates (50 μ L/well), and incubated for 12 h at 4°C. Ten grams per liter BSA were used as a control. After abandoning remnant liquid, no-serum culture solution that contained 10 g/L BSA (50 μ L/well) was added and incubated at 37°C for 30 min. Tumor cells were digested with 2.5 g/L pancreatic enzyme and modulated to a density of 1×10^5 cells/mL. One hundred microliters of cell suspension was seeded in invested Matrigel per well. Every group had four samples at equal pace. The cells were cultivated with RPMI 1640 medium that contained 10 g/L BSA and 10% fetal bovine serum for 1 h at 37°C, then the absorbance (A) was determined by the MTT colorimetric method. Cell adhesion rate of the Matrigel group was calculated by the following formula:

$$\text{Adhesion rate} = (A_{\text{exp}}/A_{\text{BSA}} - 1) \times 100\% \text{ equation 1}$$

In vitro invasion and migration assay

Invasion assays were done in a Boyden chamber with polyethylene terephthalate filter inserts for 24-well plates containing 8- μ m pores (Becton Dickinson Labware, NY, USA). Briefly, after coating the filter with 100 μ L 1:3 diluted Matrigel (Becton Dickinson) overnight at 4°C, cells were seeded in the upper chamber at a final concentration of 1.0×10^5 /mL in serum-free medium with 0.1% BSA. Eight hundred microliters medium conditioned with 10 μ g/mL fibronectin was placed in the lower compartment of the chamber as a chemoattractant. After 24 h incubation, the remaining tumor cells on the upper surface of the filters were removed by wiping with cotton swabs, and the invading cells on the lower surface were stained with hematoxylin-eosin. The invading cells on the underside of the membrane were photographed and counted under a microscope at a magnification of $\times 200$. We performed four individual experiments using the invasion assay in triplicate. *In vitro* migration assays were done under the same conditions as the invasion assays, but in non-Matrigel-coated chambers.

Establishment of an experimental metastasis model in nude mice

Cells were digested with 2.5 g/L pancreatic enzymes, washed with no-serum culture solution, and centrifuged at 1800 r/min. The cell sediment was washed with serum-free culture solution, centrifuged twice and floated in sterile PBS solution. Two hundred microliters of cell suspension

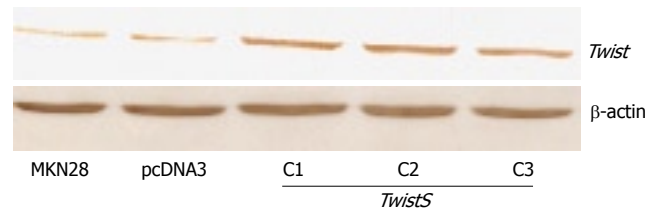


Figure 1 Western blot analysis of *Twist* expression in MKN28, pcDNA3 control and *TwistS* transfectant clones (C1, C2 and C3).

that contained 1×10^7 MKN28, pcDNA3-MKN28 and *TwistS*-MKN28 cells was seeded into the abdominal cavity of nude mice by syringe. The vim, appetite and defecation of nude mice were observed regularly, and their weight was recorded. After 9 wk, nude mice were killed and examined.

Gelatin zymography assay

MKN45, pcDNA3-MKN45 and *TwistAS*-MKN45 cells were cultured. Three days later, cells were washed by D-Hanks' solution, and then seeded into serum-free culture solution. Twenty-four hours later, the supernatant was collected and concentrated with Amicon filters (Millipore) to a 10% initial volume. Each sample was guaranteed to contain the same amount of total protein. Gelatin zymography was performed in 10% (w/v) polyacrylamide that contained 0.1% (w/v) gelatin. The identification of transparent bands at 62 kDa on the Coomassie blue background of the slab gel was considered positive for the presence of enzymatic activity.

Statistical analysis

Results were expressed as mean \pm SD for experiments with triplicate measurements. Differences between groups were tested with Student's *t* test and $P < 0.05$ was considered significant.

RESULTS

Gene transfection

MKN28 cell clones transfected with pcDNA3 or pcDNA3-*TwistS* were obtained after gene transfection. Expression of *Twist* protein was increased in the three positive cell clones that were transfected with pcDNA3-*TwistS* (Figure 1). To avoid the deviation brought about by a single cell clone, three positive cell clones were used simultaneously.

Overexpression of Twist in MKN28 cells promoted adherence, migration and invasion ability

A value was decided by the MTT colorimetric method and cell adhesion rate was calculated by equation 1. Compared with that of MKN28 (23.5%) and pcDNA3-MKN28 cells (24.2%), the adherence rate of *TwistS*-MKN28 cells (52.8%) was obviously increased ($P < 0.05$) (Figure 2A). Compared with that of MKN28 (22) and pcDNA3-MKN28 cells (25), the migration rate of *TwistS*-MKN28 cells (54) clearly increased ($P < 0.05$) (Figure 2B). Compared with that of MKN28 (40) and pcDNA3-MKN28 cells (36), the invasion rate of *TwistS*-MKN28 cells (62) also clearly increased ($P < 0.05$) (Figure 2C). Every sample was

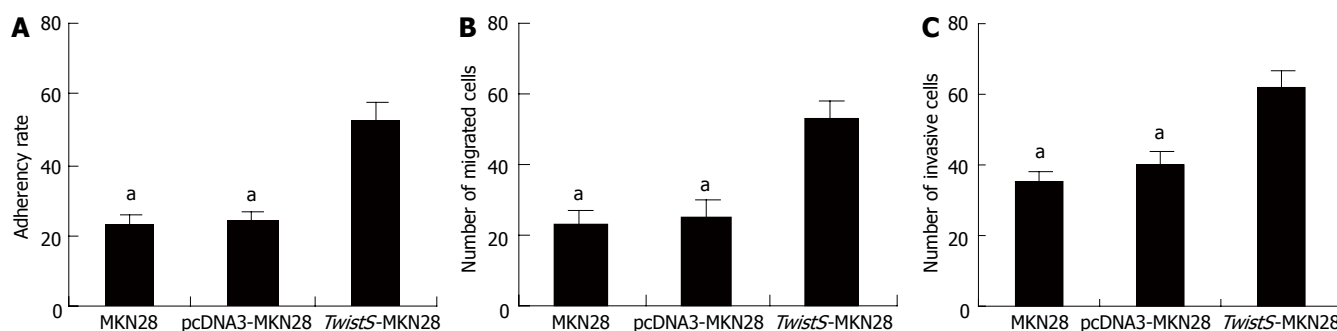


Figure 2 Cell adherence rate, and cell migration and cell invasion ability. **A:** Adherence rate in three cell lines; **B:** Migration ability in three cell lines; **C:** Invasion ability in three cell lines. ^a*P* < 0.05 vs *TwistS*-MKN28 cells.



Figure 3 Livers from nude mouse models of gastric cancer cell metastasis inoculated with: (A) MKN28; (B) pcDNA3-MKN28; (C) *TwistS*-MKN28 cells.

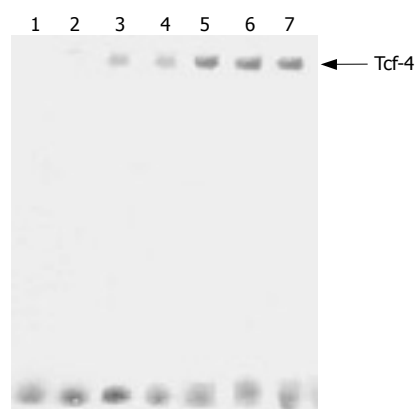


Figure 4 *Twist* promoted Tcf-4/Lef DNA binding activity with EMSA. Lane 1: Free probe; Lane 2: Competitor; Lane 3: MKN28 cells; Lane 4: pcDNA3 control cells; Lanes 5-7: Three *TwistS* clones.

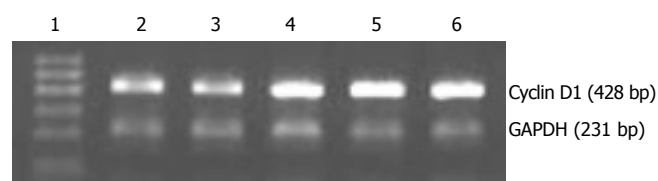


Figure 5 *Twist* increased expression of cyclin D1 mRNA by RT-PCR. Lane 1: Marker; Lane 2: MKN28 cells; Lane 3: pcDNA3 control cells; Lanes 4-6: Three *TwistS* clones.

counted for five different visual fields.

Establishment of an experimental metastasis model in nude mice

The metastasis models of gastric carcinoma cells were established in fifteen nude mice. Eight weeks later, the mice were killed and underwent exploratory laparotomy. Number and size of metastatic nodules were calculated and measured. There were lots of bigger cancer nodules in the abdominal cavity and liver of the nude mice inoculated with *TwistS*-MKN28 cells, while fewer nodules were present in the nude mice inoculated with MKN28 and pcDNA3-MKN28 cells (Figure 3).

Overexpression of *Twist* in MKN28 cells increased Tcf-4/Lef DNA binding activity

Consistent with the increasing expression levels of *Twist*,

induction of Tcf-4/Lef binding activity was observed in MKN28 and pcDNA3 control cells. Moreover, Tcf-4/Lef DNA binding was significantly increased in *TwistS* transfectants (Figure 4).

Overexpression of *Twist* promoted expression of cyclin D1 and mmp-2

We explored whether overexpression of *Twist* affected expression of cyclin D1 and mmp-2. By RT-PCR, cyclin D1 RNA levels were markedly higher in *TwistS* cells than in MKN28 and pcDNA3 control cells (Figure 5). Western blot analysis showed that *TwistS* cells exhibited increased expression levels of MMP-2 compared with those in MKN28 cells and pcDNA3 control cells (Figure 6A).

Suppression of *Twist* inhibited invasion in the human gastric cancer cell line MKN45

To observe the invasion-promoting effect of *Twist* in gastric cancer cells, a pcDNA3/-*TwistAS* vector was transfected into MKN45 cells to generate stable transfectants. As shown in Figure 6B, *Twist* expression was



Figure 6 Western blot analysis in each cell line. **A:** Expression of MMP-2 in MKN28 cells, pcDNA3 control cells and *Twist*AS cells by Western blot analysis; **B:** Western blot analysis of *Twist* expression in MKN45, pcDNA3 control and *Twist*S transfectants clone (C1, C2 and C3).

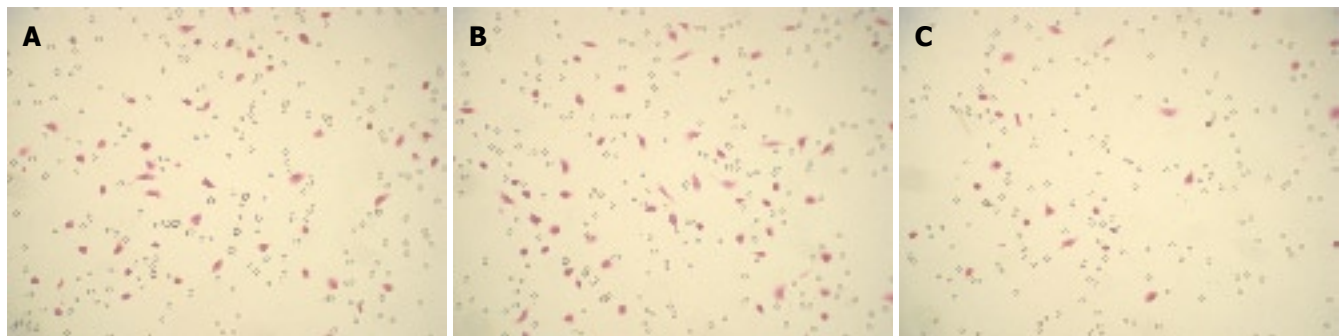


Figure 7 Down-regulation of *Twist*-inhibited invasion in MKN45 cells. Representative photographs of invasion in three cell lines (hematoxylin-eosin staining, × 200 magnification). **A:** MKN45; **B:** pcDNA3-MKN45; **C:** *Twist*AS-MKN45.

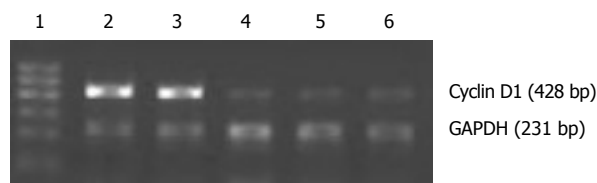


Figure 8 Suppression of *Twist*-inhibited expression of cyclin D1 mRNA by RT-PCR. Lane 1: Marker; Lane 2: MKN45 cells; Lane 3: pcDNA3 control cells; Lanes 4-6: Three *Twist*AS clones.

inhibited by *Twist*AS transfectants at the protein level. We assessed the effect of down-regulation of *Twist* on cell invasion. Representative photos showed that invasion in *Twist*AS transfectants was markedly reduced compared with that of MKN45 or pcDNA3 –MKN45 cells (Figure 7).

Suppression of *Twist* inhibited expression of cyclin D1

We investigated whether suppression of *Twist* affects the expression of cyclin D1. Cyclin D1 RNA levels were markedly lower in *Twist*AS cells than in MKN45 and pcDNA3 control cells (Figure 8).

Suppression of *Twist* inhibited gelatinase activity

Gelatin zymography revealed prominent 62 kDa bands (Figure 9), and there was activity of MMP-2 in MKN45, pcDNA3-MKN45 and *Twist*AS-MKN45 cell supernatants. Compared with that of MKN45 and pcDNA3-MKN45 cells, the activity of MMP-2 in *Twist*AS-MKN45 cells dropped markedly.

DISCUSSION

It is commonly believed that *Twist* expression is correlated

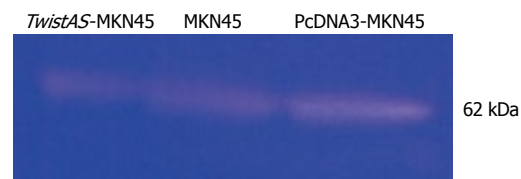


Figure 9 Comparison of activity of MMP-2 by gelatin zymography assay.

with potent invasiveness as well as poor prognosis in epithelial cancer^[8,11,13,19]. In gastric cancer, a recent report has shown overexpression of the *Twist* gene is more frequently found in diffuse-type carcinoma tissues with high N-cadherin gene expression^[15]. However, no definitive results have indicated *Twist* promotes the invasion and metastasis of gastric cancer. We have previously demonstrated endogenous *Twist* is expressed abundantly in MKN45 cells, but at much lower levels in MKN28 cells. Therefore, we transfected MKN28 cells with the *Twist* sense plasmid. Our findings suggested *Twist* probably promoted adherence, migration, invasion and metastasis of gastric cancer cells, using the MTT and Boyden chamber methods, and metastasis models of gastric carcinoma in nude mice. However, when we transfected MKN45 cells with the *Twist* antisense vector, results indicated suppression of *Twist* inhibited cell invasion. Therefore, we think *Twist* may play an important role in invasion and metastasis of gastric carcinoma.

A large number of genes relevant for tumor formation and progression have been found to be transcriptionally activated by the β -catenin/Tcf complex. Some of these are implicated in growth control and cell cycling (c-myc, cyclin D1), Some are relevant for cell survival (Id2, MDR1), one is the EMT marker vimentin, and others are

implicated in tumor invasion and metastasis (matrilysin, VEGF, cd44)^[16-18]. Tcf-4/Lef is the transcription factor for Wnt signaling. Our results indicated that overexpression of *Twist* in MKN28 cells increased Tcf-4/Lef DNA binding activity, and promoted expression of Tcf-4' downstream gene cyclin D1. The reason may be that overexpression of *Twist* redistributes β -catenin to the nucleus, in which it forms a functional transacting complex by associating with the Tcf4/Lef-1 transcription factor, and enhances the transactivation of a number of genes including cyclin D1, VEGF and EMT marker vimentin. Cyclin D1 plays an important pole in cell proliferation^[20]. Our results agreed with previous studies that showed that *Twist* promotes growth of breast cancer cells. In breast cancer MCF-7 cells, colocalization of β -catenin and E-cadherin was prominent at the plasma membrane, whereas in MCF-7 cells with overexpression of *Twist*, these proteins were distributed within the cytoplasm and to a lesser extent within the nucleus. In addition, the total amount of epithelial marker protein β -catenin and E-cadherin was lower in MCF-7 cells^[21]. This can partly explain the phenomenon of EMT by *Twist*.

Metastatic potential requires proteolytic degradation of the extracellular matrix, and MMPs are thought to play an important role in tumor invasion and metastasis. We demonstrated that overexpression of *Twist* in MKN28 cells promoted expression of mmp-2, while suppression of *Twist* in MKN45 cells inhibited the activity of mmp-2. However, to the best of our knowledge, the E-box site, which is the binding site for *Twist*, was not observed in the promoter region of the MMP gene family. One possible explanation is that MT1-MMP expression is induced through β -catenin/Tcf4 expression^[22,23], followed by increased activation of MMP-2. Another explanation is that MMP-2 activity is induced through the up-regulation of MT1-MMP expression, by inhibition of zonula occludens 1 tight junction complex expression that is changed by EMT^[24].

In conclusion, *Twist* may contribute to the invasion and metastasis of gastric carcinoma cells, mainly through EMT after regulation of Wnt signaling, or through an effect on MMP-2. At the same time, recent evidence suggests *Twist* is a major factor that participates in tumor development and progression^[25-28]. As a novel player in the invasion and metastatic program, *Twist* is gaining rapid attention^[29,30]. Our findings of a functional link between *Twist* and Tcf4/Lef-1 suggest targeting *Twist* may provide novel therapeutic cocktails for gastric cancer intervention.

ACKNOWLEDGMENTS

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COMMENTS

Background

Invasion and metastasis are the most common cause of death and is a major

obstacle to successful treatment of gastric cancer. It is necessary to develop effective new strategies for the prediction, diagnosis and treatment of gastric cancer invasion and metastasis.

Research frontiers

Recent investigations have shown *Twist* is elevated in prostate and breast cancer. High expression of *Twist* is positively related to cancer invasion and metastasis, but few studies have investigated *Twist* expression in gastric cancer, and the impact of *Twist* on prognosis.

Innovations and breakthroughs

Twist promotes gastric cancer cells migration, invasion and metastasis, and *Twist* may play an important role in Wnt/Tcf-4 signaling.

Applications

Our finding of a functional link between *Twist* and Tcf4/Lef-1 suggests that targeting *Twist* may provide novel therapeutic cocktails for gastric cancer intervention.

Terminology

EMT is a process whereby epithelial cells lose polarity and cell-to-cell adhesion, and undergo dramatic remodeling of the cytoskeleton. Concurrent with loss of epithelial cell adhesion and cytoskeletal components, cells undergoing EMT acquire expression of mesenchymal components and a migratory phenotype.

Peer review

This study was well designed and may be important for providing additional evidence to support the role of *Twist* expression in promoting tumor metastasis, including in gastric cancer.

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BASIC RESEARCH

Effect of 5-LOX/COX-2 common inhibitor DHDMBF30 on pancreatic cancer cell Capan2

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the pancreatic cell line Capan2, and induces apoptosis and inhibits the growth of pancreatic cancer in nude mice.

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Key words: 5-lipoxygenase pancreatic tumor Cyclooxygenase2; DHDMBF30; Carcinoma in nude mice

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Zhang B, Wang CL, Zhao WH, Lv M, Wang CY, Zhong WX, Zhou WY, Yu WS, Zhang Y, Li S. Effect of 5-LOX/COX-2 common inhibitor DHDMBF30 on pancreatic cancer cell Capan2. *World J Gastroenterol* 2008; 14(16): 2494-2500 Available from: URL: <http://www.wjgnet.com/1007-9327/14/2494.asp> DOI: <http://dx.doi.org/10.3748/wjg.14.2494>

Abstract

AIM: To study the effect of 5-lipoxygenase/cyclooxygenase-2 (5-LOX/COX-2) dual inhibitor 7-tert-butyl-2, 3-dihydro-3, 3-dimethyl substituted dihydrofuran 30 (DHDMBF30) on proliferation and apoptosis of the pancreatic cancer cell line Capan-2 and the effect of DHDMBF30 on human pancreatic cancer in a nude mouse model.

METHODS: Investigate the effect of 5-LOX/COX-2 dual inhibitor DHDMBF30 on proliferation and apoptosis of the pancreatic cancer cell line Capan-2 by RT-PCR, MTT assay, FCM and electron microscope. Cell line Capan-2 was inoculated percutaneously on the outer thigh of 12 nude mice. The VEGF mRNA of transplantation tumor was detected by RT-PCR.

RESULTS: DHDMBF30 inhibits the proliferation of cell line Capan2, reduces the expression of 5-LOX, COX-2 and VEGF. After Capan2 was treated with DHDMBF30, we found that the apoptosis peak of the experimental group was significantly higher than that of the contrast group (3.08 ± 1.89 vs 27.67 ± 0.52 , $P < 0.001$). The tumor weight of the DHDMBF30 group was significantly lower than PBS control groups (1.35 ± 0.47 vs 2.92 ± 0.73 , $P < 0.01$). Expression of VEGF in the DHDMBF30 group was significantly decreased.

CONCLUSION: DHDMBF30 inhibits the proliferation of

INTRODUCTION

Through many years of practice and research, the treatment of pancreatic cancer has made some advancement in the areas of surgical operation, radiotherapy and chemotherapy, but the therapeutic effect is not yet satisfied enough, especially in the patients of advanced stages, and the remission rate or survival rate has not been improved significantly. At present, the major treatment method is the combined therapy, such as surgical operation combined with chemotherapy and (/or) radiotherapy *etc.* The prevention and treatment mechanism of non-steroid anti-inflammatory drug (NSAIDs) against tumors are still not clear now, but it is certain that NSAIDs can inhibit cyclooxygenase-2 (COX-2), which is the key enzyme in the synthesis of prostaglandin (PG). Some experiments demonstrated that the selective COX-2 inhibitors could inhibit the tumor cell proliferation, induce apoptosis and inhibit the generation of new vessels for tumors^[1,2]. In recent years, a number of research studies have demonstrated that NSAIDs could inhibit 5-lipoxygenase (5-LOX), thereby inhibiting proliferation and inducing apoptosis of many malignant tumors^[3,4]. In our early phase study, it was demonstrated that the expression rates of 5-LOX and COX-2 in the pancreatic cancer tissues were respectively 74.3% and 80%^[5,6], and all the pancreatic cancer tissues expressed 5-LOX or COX-2, sometimes expressed both, which supported that

there were crossed and complementary expressions, so the designed 5-LOX/COX-2 dual inhibitor can exert a synergistic effect. So we chose the 5-LOX/COX-2 dual inhibitor DHDMBF30 to act against pancreatic cancer cells, and observed the anticancer effects in order to provide a foundation for its clinical application.

MATERIALS AND METHODS

Materials

The human pancreatic cancer cell Capan-2 was obtained from the Pathology Teaching Research Department of Peking Union Medical College Hospital; pancreatic cancer cell medium RPMI1640 (GIBCO company) contained 20 mmol/L NaHCO₃, 100 U/mL penicillin, 100 U/mL streptomycin, 10% calf serum (Hangzhou Sijiqing Biological Engineering Materials Co, Ltd); 0.25% trypsin (Sigma company). The primers were synthesized by the Shanghai Institute of Biochemistry, Chinese Academy of Sciences, RNA extract reagent Trizol[®] and RT-PCR kits were bought from GIBCO company. DHDMBF30 was bought from Procter & Gamble Pharmaceuticals (Ohio). Experimental animals: BALB/C nu hairless mice were bought from the Institute of Laboratory Animal Science, Chinese Academy of Medical Sciences. The mice were 4-6 wk old, and there were 6 males and 6 females.

Cell culture

Pancreatic cancer cell strain Capan-2 was cultured in RPMI1640 medium containing 20% calf serum, and the cell strain was generally cultured in 37°C incubator with 5% CO₂. Digestion of the strains was performed with 0.25% trypsin and serially sub-cultivated after 70%-80% of them coalesced during the adherent growth process. Next, we inoculated Capan-2 cells into the 100 mL culture flask at a dose of 10⁶/flask after the strains were digested, then divided the strains into control group and experimental group, cultured for 3 d, and changed the medium when the cells entered into the exponential division phase, then added 12 µmol/L DHDMBF30 into the culture flask in the experimental group and cultured for another 24 h.

The experimental animals were randomly divided into two groups

Six mice in each group were put into a rearing cage. Experimental group: on the second day after cell inoculation each mouse was subcutaneously injected with 0.2 mL (12 µmol/L) DHDMBF30 in the inoculation area of tumor cells twice a week for 3 wk; PBS control group: the injection time, site and volume were the same as the treatment group. After the tumor could be touched, we measured the long and short diameters of the tumor every 3 d. Hairless mice were sacrificed 35 d later, the tumor mass measured and tissue fixed with 10% formalin. Immunohistochemistry and pathological examinations were then performed.

Detect the cell inhibition ratio by MTT method

Cells were cultured in 1640 liquid culture media containing

20% calf serum, and transferred into 1640 liquid without calf serum after 24 h, prepared at a 5×10^4 /mL cell suspension and added into a 96 well plate with 0.1 mL suspension in each well. For every dose group, a set of 3 auxiliary wells were prepared and we added DHDMBF30 of different concentrations, set the final concentrations at 5 µmol/L, 10 µmol/L, 15 µmol/L, and 20 µmol/L with the total volume of 100 µL and continued to culture the cells. Added 20 µL 5 mg/mL MTT 4 h before stopping drug at 12, 24, 36 and 48 h, then returned the culture plate into the CO₂ incubator at 37°C for 4 h (the cells would deoxidize the yellow MTT to hyacinthine crystallization under the action of SDH). Next we aspirated and discarded 90% of the culture liquid in the wells, added 100 µL DMSO into each well and thoroughly shook the wells for 10 min to completely dissolve the crystals. We determined the A values of each well at the wavelength of 570 nm, and calculated the GIR of the cells. We repeated the experiment 6 times. Calculated GIR with the formula:

$$\text{GIR (\%)} = \frac{\text{A value of control pore} - \text{A value of experimental pore}}{\text{A value of the control pore}} \times 100\%$$

Detect the Capan-2 cell apoptosis with flow cytometry

After the cells were cultured in the 1640 liquid culture media containing 20% calf serum for 24 h, the liquid was replaced with the 1640 liquid culture media without calf serum for another 24 h culture, and finally the cells were digested with 0.25% trypsin and 0.02% EDTA to prepare a unicell suspension with the cell concentration at about 5×10^5 /mL. The suspension was plated into a 6-well plate with 1 mL suspension in each well. The control group was drug free; 12 µmol/L DHDMBF30 was added to the experimental group and cultured for 24 h again. We repeated this experiment 3 times. Suspensions were also prepared and stained with propidium iodide (PI), then injected the suspension.

Sample preparation for transmission electron microscopy

Cells from the control and experimental groups were fixed, dehydrated, replaced, soaked and embedded, prepared semi thin sections, stained with electrons and observed for ultra structural structures.

RT-PCR

Process: (1) Collect the cells from the control and experimental groups: take cells cultured for 24 h, digest with 0.25% trypsin to prepare unicell suspension, centrifuge at 500 r/min for 10 min, collect the cell precipitates and rinse with 0.01mmol/L PBS (pH = 7.4) for one time, then store at -70°C; (2) Extraction of total RNA of the cell: extract the total RNA with the Trizol[®] kit, learn about the integrity of the extracted RNA by formaldehyde denaturing gel electrophoresis, determine $A_{260 \text{ nm}}$ and $A_{280 \text{ nm}}$ values by ultraviolet spectrophotometer to analyze its purity, and fix quantity at the same time; (3) The synthesis of cDNA: mix 1 µg RNA sample with 1.2 µL random hexamer primer, put them into an iced bath after annealing at 70°C for 5 min, then add 4 µL reverse transcriptase buffer, 1 µL 20-40 Mu/L Rnasin and 2 µL 10 mmol/L dNTP, mix them completely at 25°C for 5 min; add 2 µL MmuLV reverse transcriptase finally and add water to 20 µL volume, heat at 25°C for

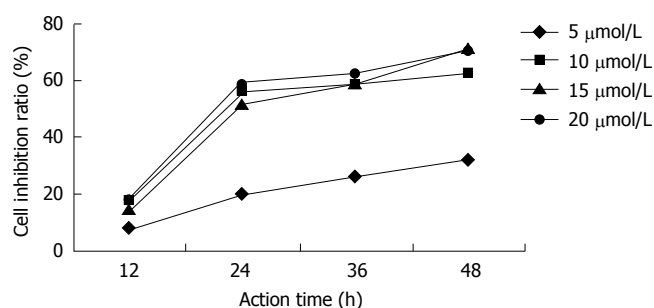


Figure 1 Effect of DHDMBF30 against the Capan-2 cell proliferation of human pancreatic cancer.

5 min, 37°C for 60 min and 70°C for 10 min, stop the reaction and store the reagents at 4°C; (4) PCR: the primer sequence of 5-LOX was 5'-CCCGGGGCATGGAGAGCA-3', 5'-GCGGTCGGGCAGCGTGTC-3'; COX-2: 5'-TTCA AATGAGATTGTGGGAAAATTGCT-3', 5'-AGATCATC TCTGCCTGAGTATCTT-3'; VEGF: 5'-TTGCTGCTCTA CCTCCAC-3', 5'-AATGCTTTCTCCGCTCTG-3'; β -actin: 5'-GTGGGGCGCCCCAGGCACCA-3', 5'-CTCCTTAAT GTCACGCACGATTT-3'. The lengths of the amplification fragments were 416, 305, 418, 500 bp respectively; the reaction systems were 0.8 μ L cDNA, 2 μ L 10 \times Taq enzyme buffer, 0.4 μ L 10 mol/L dNTP, 0.8 μ L 25 mmol/L $MgCl_2$, the up and down stream primers were 0.4 μ L and 0.5 μ L Taq enzymes, and the water was added to 20 μ L. PCR conditions: denature at 95°C for 45 s, anneal at 54°C for 90 s, elongate at 72°C for 90 s, and repeat for 30 cycles. At last, elongate at 54°C for 2 min and 72°C for 3 min. After the reaction, take out 10 μ L amplification products to 17 g/L agarose gel for electrophoretic analysis. In order to compare the intensities of expression levels, scan the amplification bands by image analyzer for semi-quantitative analysis.

We detected the VEGF mRNA of the transplanted tumor by RT-PCR, and the VEGF protein by the immunohistochemistry SP method. We measured the absorbance of the immunohistochemistry stained slide by microspectrophotometer, chose 100 cells randomly in each slide, scanned the absorbance value of each cell at the wavelength of 460 nm and calculated the expression area, worked out their means to express the relative content of VEGF protein.

RESULTS

The effect of DHDMBF30 against the Capan-2 cell proliferation of human pancreatic cancer

After the actions of DHDMBF30 of different concentrations against Capan-2 cells for 12 h, 24 h, 36 h, 48 h, with the concentration increasing and the duration prolonging, the inhibition was strengthened, but after the concentration and the prolonged duration reached certain values, the inhibition ratio didn't increase anymore, but a plateau appeared, the IC_{50} was 12 μ mol/L and the inhibition ratio didn't increase after 24 h (Figure 1).

Detect the Capan-2 cell apoptosis rate with flow cytometry

There was an apoptosis peak of Capan-2 cells (3.08 ± 1.89)

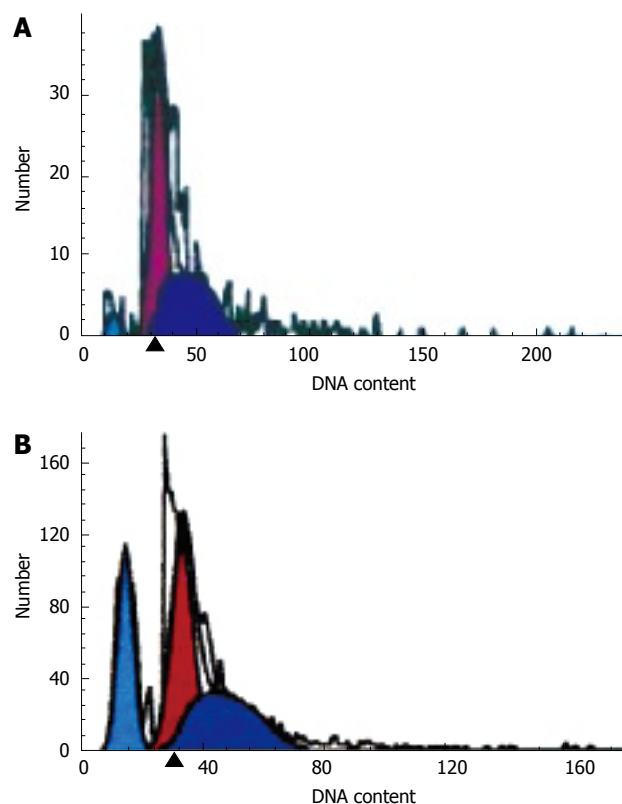


Figure 2 Apoptosis rate of Capan-2 cells. A: Before the treatment of DHDMBF30; B: After the treatment of DHDMBF30.

before G_0/G_1 , and the peak (27.67 ± 0.52) was higher after the treatment by DHDMBF30, which was significantly different compared with the control group ($P < 0.001$) (Figure 2).

Observation by transmission electron microscope

The Golgi's complexes in the Capan-2 cells of the control group were developed, there were a lot of rough endoplasmic reticula, chondrosomes were swelling, the karyoplasmic ratio was high, the karyotheca showed depressed wrinkles, and the chromatin were abundant in the nucleus and nucleoli were large. After a 12 h treatment with DHDMBF30, microvilli of most of the cell surface were reduced, the cell volume was decreased and the cytoplasm concentrated, a large number of vacuolar degenerations appeared in some cells; the cell nucleus was shrunk, the karyotheca existed but the nucleolus disappeared, the dyeing of chromatin was darkened and the latter congregated into masses adjoining the karyotheca; 24 h later, the membrane became smooth and the cytoplasm continued to condense, there were concentrated nuclear fragments, and some cells produced apoptotic bodies through budding. The following figures show the nuclear fragments enclosed by membrane structures and degenerated cell organs (Figure 3).

The expressions of 5-LOX and COX-2 genes in the pancreatic cancer Capan-2 cells and the inhibition of DHDMBF30 against them

In the electrophoretic analysis on RT-PCR products of pancreatic cancer cell strains in the control group, one

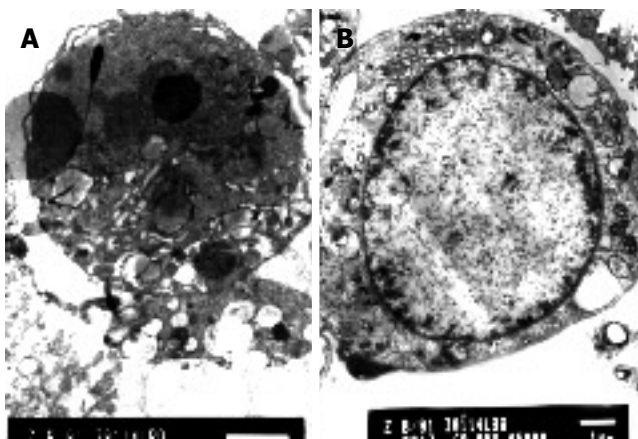


Figure 3 Observation by electron microscope. **A:** Before the treatment of DHDMBF30 on Capan-2 cells; **B:** After the treatment of DHDMBF30 on Capan-2 cells.

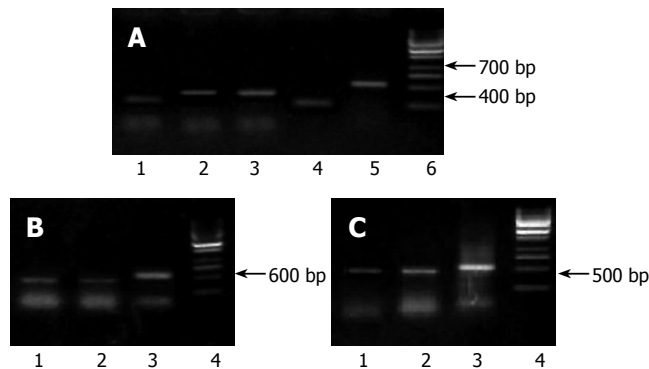


Figure 4 The effect of DHDMBF30 against different mRNA. **A:** Against 5-LOX mRNA and COX-2-mRNA in Capan2 cells. Lane 1: COX-2 after inhibition; Lane 2: 5-LOX after inhibition; Lane 3: 5-LOX before inhibition; Lane 4: COX-2 before inhibition; Lane 5: β -actin; Lane 6: Marker; **B:** Against VEGF mRNA in Capan2 cells. Lane 1: Control group; Lane 2: Treatment group; Lane 3: β -actin; Lane 4: Marker; **C:** Against the VEGF mRNA of transplant pancreatic tumor cell in the hairless mice. Lane 1: Treatment group; Lane 2: Control group; Lane 3: β -actin; Lane 4: Marker.

416 bp and one 304 bp amplification bands could be observed; the 416 bp and 304 bp amplification bands also existed in the experimental group, but the brightness was lowered (Figure 4A), which demonstrated that there were expressions of 5-LOX and COX-2 genes in the Capan-2 cells of pancreatic cancer, and the inhibitor decreased their expressions.

The effect of DHDMBF30 against VEGF mRNA in Capan2 cells of the human pancreatic cancer

A 418 bp band was obtained in the electrophoretic analysis of RT-PCR products from the pancreatic cancer cell strains in the control group, and there was also a specific 418 bp amplification band with a lower brightness in the experimental group (Figure 4B).

The growth conditions of the transplant tumor: The subcutaneous tumor began to form 2-3 wk after the inoculation, and the volume reached 2-3 cm 4-5 wk later. In the PBS control group, the transplant tumor formed in all the inoculation regions, and all of the tumor volumes

Table 1 The inhibition of DHDMBF30 against the transplant human pancreatic tumor in the hairless mice

Groups	Tumor formation rate (%)	Tumor weight (g)
DHDMBF30 group	67 (4/6)	1.35 ± 0.47^b
PBS group	100 (6/6)	2.92 ± 0.73

^b $P < 0.01$ vs PBS group.

Table 2 The effect of DHDMBF30 against VEGF proteins of the transplant pancreatic tumor

Groups	Absorbance	Expression area (μm^2)
Experimental group	36.36 ± 7.23^b	45.82 ± 9.67^b
Control group	66.61 ± 9.63	73.06 ± 8.45

^b $P < 0.01$ vs control group.

were large, the tumor formulation rate was 100% (6/6); while the formulation rate in the experimental group was 67% (4/6) and the volume was small. The subcutaneous tumors grew slowly and the tumor bodies were hard, they were all fixed on the lateral side of the thigh. When the diameter of the tumor body reached 25 mm in the advanced stage, gross nutrition vessels could be observed on the skin upon the tumor body.

The growth of transplant tumor under the skin of the hairless mice and the change of tumor weight: the growth rate of the transplant pancreatic tumor in hairless mice of the experimental group was obviously lower than the PBS control group; the tumors could be touched on the 14th d in the PBS group, while it was the 20th d in the experimental group. The average weight of the hairless mice in the experimental group was (1.50 ± 0.52) g, which was significantly lower than (2.18 ± 0.96) g of the PBS control group ($P < 0.01$) (Table 1).

Detection of VEGF mRNA and proteins

The detection results of VEGF mRNA: The 418 bp band amplified in transplant tumor indicated the expression of the VEGF mRNA, and the brightness of VEGF mRNA band in the DHDMBF30 treating group was obviously lowered (Figure 4 C).

The expression of VEGF protein: The results of immunohistochemistry showed that the VEGF expression was positive in the transplant human pancreatic tumor in the hairless mice of the control group. Positive buffy granules could be seen in the cytoplasm of the pancreatic cancer cells; the cytoplasm of pancreatic cancer cells of the DHDMBF30 group stained lightly, and the stained cell number was small, so it was weakly positive. Please refer to the table for the comparison of absorbance and expression area (Figure 5; Table 2).

DISCUSSION

NSAIDs mainly act against the mechanism of arachidonic acid (AA) to produce a marked effect. There are two major

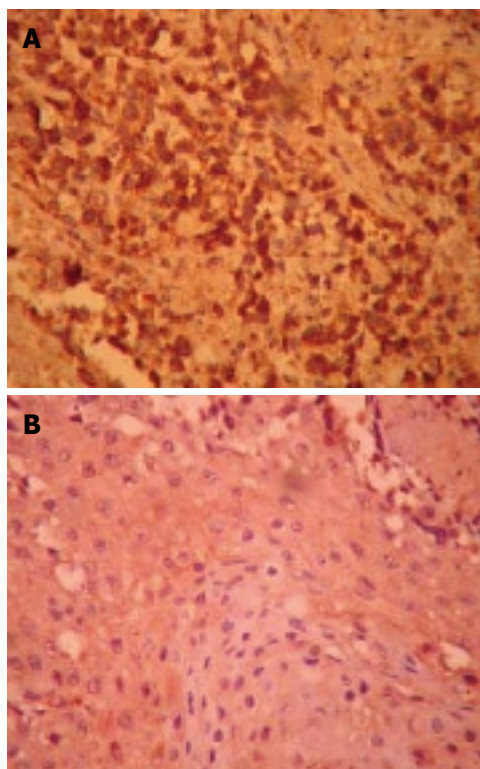


Figure 5 The VEGF expression of different group. **A:** Control group; **B:** DHDMBF30 group.

metabolic enzymes of AA: lipoxygenase (LOXs) and cyclooxygenase (COXs)^[7,8]. It has been found in a large number of studies in recent years that, 5-LOX and COX-2 of the lipoxygenase and cyclooxygenase can not only act against infections, but also relate to the pathogenesis, development and transfer of the tumors^[9-14]. Luo *et al*^[15] reported that 5-LOX in the endothelial cells could promote the generation of cancer, colonitis, psoriasis, *etc*. Ohno, *et al*^[16-19] determined the expression quantity of COX-1 and COX-2 of the surgical sample obtained from gastric cancer in 33 patients by RT-PCR, stained the COX-2 antibody in the sample with immunohistochemistry staining and did routine histology examination at the same time, and they found that the COX-2 expression index in the gastric cancer was significantly higher than the normal mucosa (3.4-0.7 *vs* 2.2-0.7, $P < 0.05$), and the index of COX-2 increased with the infiltration of gastric cancer advancing.

Tenidap developed by Pfizer was a drug with a double inhibition effect against COX/5-LOX, and it was demonstrated in the clinical trials that its therapeutic effect was superior over other NSAIDs, such as diclofenac sodium, Naproxen, *etc*, but its hepatotoxicity was high, so it was withdrawn before long it came into the market. There was still a study in which the effect of the combination of COX-2 and 5-LOX inhibitors against the tumor was observed. At present, the researchers dedicated themselves to research and develop the selective 5-LOX/COX-2 double inhibitors, the DHDMBF had a certain double inhibition effect^[20-22], through modifying the cycles or sites, researchers obtained more than 30 chemicals, among which the effect of DHDMBF30 was

good both *in vivo* and *in vitro* and its inhibition ratio against COX-1 was extremely low, but there was no study on its effect against tumors. We found in our study that, 24 h after DHDMBF30 of different concentrations acted against Capan-2 cells, the inhibition ratio of the higher concentration group to Capan-2 was obviously increased; after the DHDMBF30 treatment on Capan-2 cells, the cellular morphologies were obviously altered, the granules and vacuoles were increased, some cells contracted and became rounder, the cell membrane shrunk, many cells dropped from the glass wall and were suspended in the culture liquid, and the dropped cells gradually increased with duration in culture and increasing drug concentration. Twenty-four hours later, the cellular membrane became smoother and the cytoplasm continued to condense, concentrated nuclear fragments could be observed and apoptotic bodies were produced in some cells through budding. In the detection of Capan-2 cell apoptosis by flow cytometry, it was found that there was an apoptosis peak of the Capan-2 cells in the control group before G₀/G₁, and the peak was heightened after the treatment of DHDMBF30, the results were significantly different compared with the control group^[23,24].

In former studies, COX-2 or 5-LOX inhibitors were separately applied to interfere in the tumor cells, and their effects were observed. Recently, some researchers have combined selective COX-2, 5-LOX inhibitors or broad-spectrum inhibitors to act against the tumor cells to maximally kill the tumor cells, but with increasing dose, the side effects were certainly increased. Thereby, we applied one drug to observe its effect in order to obtain the optimal therapeutic effect. As a common clinically applied and symptomatic treating drug for arthritis, the side effects of NSAIDs in the gastrointestinal tract were common, so the pharmaceutical chemists at home and abroad had always tried to find a new anti-inflammatory agent of high efficiency and low toxicity. The inhibiting concentration ratio (IC₅₀) of NSAIDs against COX-2/COX-1 could reflect the side effects of the drug for the applied anti-inflammatory dose: if the COX-2/COX-1 IC₅₀ was less than 1, the drug could selectively inhibit COX-2, the anti-inflammatory effect was strong while the side effects on stomach and kidney were less; if the COX-2/COX-1 IC₅₀ was more than 1, the drug could strongly inhibit COX-1 with more side effects. Garcia Rodrigues and Jick^[25] publicized epidemiological statistics data of the side effects of NSAIDs, the results confirmed that the inhibition concentration ratio of COX-2/COX-1 and side effects of gastrointestinal tract had a parallel relationship. The selective COX-2 inhibitor meloxicam, which had been registered in our country and some other countries, was designed to treat rheumatoid arthritis and ostarthritis, it had stronger anti-inflammatory effects and fewer side effects, and its inhibiting concentration ratio to COX-2/COX-1 was 0.07. The DHDMBF30, which we chose, was synthesized in recent years, its inhibiting concentration ratio to COX-2/COX-1 was 0.03, and the effect was good in both *in vivo* and *in vitro* experiments^[26-30].

By using RT-PCR, we confirmed Capan2 cell expressed 5-LOX mRNA, COX-2 mRNA and VEGF mRNA,

and the expression of 5-LOX mRNA, COX-2 mRNA, VEGF mRNA was obviously decreased after the treatment of DHDMBF30; morphologic changes of shrinkage and apoptosis were also observed by microscope and electron microscopy; flow cytometry detected that the apoptosis rate of Capan2 cells was significantly increased. DHDMBF30 could inhibit the proliferation of Capan2 cells and induce its apoptosis, down regulate the vascular endothelial growth factors; its effect was better than the single application of COX-2 or 5-LOX inhibitor, and it could make up the ineffective result if the 5-LOX or COX-2 was not expressed.

The tumor formation rate, the weight and volume of the tumor body of the hairless mice in the DHDMBF30 treating group were all significantly less than those of the PBS control group, and no side effects were observed at the same time. The VEGF expression of the transplant pancreatic tumors in hairless mice was decreased, and the division, growth and assorting effects on the vascular endothelial cells in the tissues were lowered. Meanwhile, it inhibited the increase of vasopermeability and decreased the exudation of matrix ingredients such as blood plasma from the tissue, the foundations on which the vascular endothelial cells and tumor cells relied were eliminated, and thereby the goal of tumor growth inhibition was achieved.

The regional injection of DHDMBF30 could inhibit the growth of pancreatic cancer, and the manifestations were the prolonged incubating stages of the tumor and the lighter tumor weight ($P < 0.01$). But DHDMBF30 still could not completely inhibit the tumor growth, which might be related to the dose, and it was demonstrated that the dose-effect relationship and time-effect relationship should be further studied.

COMMENTS

Background

The prevention and treatment mechanism of non-steroid anti-inflammatory drug (NSAIDs) against tumors are still not clear now. In our early phase study, it was demonstrated that the expression rates of 5-lipoxygenase (5-LOX) and cyclooxygenase-2 (COX-2) in the pancreatic cancer tissues were respectively 74.3% and 80%^[5,6], and all the pancreatic cancer tissues expressed 5-LOX or COX-2, sometimes expressed both, which supported that there were crossed and complementary expressions, so the designed 5-LOX/COX-2 dual inhibitor can exert a synergic effect.

Research frontiers

It has been found in a large number of studies in the recent years that, 5-LOX and COX-2 of the lipoxygenase and cyclooxygenase can not only act against infections, but also relate with the pathogenesis, development and transfer of the tumors^[9-14]. At present, the researchers dedicated themselves to research and develop the selective 5-LOX/COX-2 double inhibitors, the 7-tert-butyl-2,3-dihydro-3,3-dimethyl substituted dihydrofuran (DHDMBF) had certain double inhibition effect^[20-22], through modifying the cycles or sites, researchers obtained more than 30 chemicals, among which the effect of DHDMBF30 was good both *in vivo* and *in vitro* and its inhibition ratio against COX-1 was extremely low, but there was no study on its effect against tumors.

Innovations and breakthroughs

DHDMBF30 inhibits the proliferation of Capan2 cells and induces its apoptosis, down regulates the vascular endothelial growth factors; its effect was better than the single application of COX-2 or 5-LOX inhibitor, and it could make up the ineffective result if the 5-LOX or COX-2 was not expressed.

Applications

The regional injection of DHDMBF30 could inhibit the growth of pancreatic cancer, and the manifestations were the prolonged incubating stages of the tumor and the lighter tumor weight ($P < 0.01$). We chose the 5-LOX/COX-2 dual inhibitor DHDMBF30 to act against pancreatic cancer, and observed its anticancer effects.

Terminology

NSAIDs: Non-steroid anti-inflammatory drug. DHDMBF: The selective 5-LOX/COX-2 double inhibitors, the 7-tert-butyl-2, 3-dihydro-3, 3-dimethyl substituted dihydrofuran.

Peer review

This article is interesting which studied the effect of a kind of 5-LOX/COX-2 dual inhibitor on proliferation and apoptosis of pancreatic cancer cell line Capan2 and the effect of it on human pancreatic cancer in nude mice model. It provided a foundation for its clinical application.

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Identification of osteopontin as the most consistently over-expressed gene in intrahepatic cholangiocarcinoma: Detection by oligonucleotide microarray and real-time PCR analysis

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a specific gene expression profile was obtained allowing fast and reproducible differentiation of CCC, which was confirmed by supervised neuronal network modelling. The most consistently overexpressed gene (median fold change 33.5, significantly overexpressed in 100%) encoded osteopontin. Furthermore, an association of various genes with the histopathological grading could be demonstrated.

CONCLUSION: A highly specific gene expression profile for intrahepatic CCC was identified, allowing for its fast and reproducible discrimination against non-malignant liver tissue and other liver masses. The most overexpressed gene in intrahepatic CCC was the gene encoding osteopontin. These data may lead to a better understanding of human cholangiocarcinogenesis.

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Key words: Cholangiocarcinoma; Oligonucleotide arrays; Osteopontin; Cell cycle regulation; Gene expression

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Abstract

AIM: To investigate the molecular pathways involved in human cholangiocarcinogenesis by gene expression profiling.

METHODS: Oligonucleotide arrays (*Affymetrix* U133A) were used to establish a specific gene expression profile of intrahepatic CCC in comparison to corresponding non-malignant liver tissue. To validate the expression values of the most overexpressed genes, RT-PCR experiments were performed.

RESULTS: Five hundred and fifty-two statistically differentially expressed genes/ESTs (221 probes significantly up-regulated, 331 probes down-regulated; $P < 0.05$; fold change > 2 ; $\geq 70\%$) were identified. Using these data and two-dimensional cluster analysis,

INTRODUCTION

Cholangiocarcinoma (CCC) is the second most common primary hepatic malignancy next to hepatocellular carcinoma (HCC), arising from cholangiocytes of intra- or extrahepatic bile ducts. In contrast to HCC, cholangiocarcinomas are, in most cases, adenocarcinomas and relatively hypovascularised. Two principal forms of these tumors are recognized based on the clinical presentation^[1]. The most common form is a highly desmoplastic cancer with a growth pattern characterized

by periductal extension and infiltration, leading to an early onset of jaundice as one of the first clinical signs of this disease (perihilar and distal extrahepatic CCC). The other form of CCC grows as a mass lesion within the liver, very often leading to the misdiagnosis of hepatocellular carcinoma or metastatic adenocarcinoma (intrahepatic CCC).

Despite recent progress in various imaging modalities the neoplasm lesion is, in most cases, advanced by the time of diagnosis. Thus, curative treatment options by surgical resection are limited, leading to a poor prognosis with median survival times of only three to six months, especially in patients with intrahepatic CCCs^[2,3].

Recent epidemiologic studies have shown an increasing incidence of CCC in western countries^[4,5]. In most patients with CCC, no risk factors for cholangiocarcinogenesis can be identified. However, established risk factors for ductal cholangiocarcinoma include diseases leading to chronic inflammation of the bile ducts, such as infections with *Clonorchis sinensis*, *Opisthorchis viverrini* (liver flukes) and chronic viral hepatitis, chronic intrahepatic lithiasis, primary sclerosing cholangitis (PSC), congenital diseases (Caroli disease, congenital choledochal cysts) and exposure to the radiopaque medium thorium dioxide (Thorotrast)^[6-8]. In particular, patients with PSC have an increased risk for cholangiocarcinogenesis and epidemiologic studies have shown a lifetime risk for CCC of approximately 1.5% per year of disease^[9].

Despite this knowledge about the major role of chronic inflammation in cholangio-carcinogenesis, little is known about the molecular pathways involved in CCC that lead to uncontrolled cell growth, downregulated apoptosis and, as a result, to tissue invasion.

Therefore, it is important to identify the genes involved in these molecular pathomechanisms, as this may shed light on the specific tumor biology and help to establish prognosis patterns as well as specific markers for screening in patients with a known increased risk for CCC (for example, patients with PSC). At present, the most commonly used markers in CCC include tumor antigens or products, such as CA 19-9, cytokines (for example, interleukin-6), (epi-)genetic lesions (for example, *K-ras* and p53 mutations), and metabolic products as lactate and proteases (for example, trypsinogen-2). However, none of these have been proven to be a useful diagnostic tool with high specificity and sensitivity for CCC^[9].

Today, high-throughput techniques using cDNA or oligonucleotide arrays for the simultaneous and fast analysis of thousands of genes are available, potentially leading to the identification of new markers and subclassification of various human carcinomas^[10-12]. In the present study oligonucleotide arrays (HU 133A, *Affymetrix*) containing more than 20 000 genes and expressed sequence tags (ESTs) were used to analyze gene expression in intrahepatic CCC tissues. The aim was first to detect specific genes (by comparison with genes expressed in non-malignant liver tissue) that may be helpful for discrimination between non-malignant and different malignant liver masses, and to establish a specific diagnostic genetic profile of intrahepatic CCC for clinical

routine, and second, to elucidate the genetic pathways that are typically altered in cholangiocarcinogenesis.

MATERIALS AND METHODS

Acquisition of samples

Surgical specimens were obtained from 10 patients who underwent surgical treatment with curative intention for intrahepatic CCC between 2003 and 2004 (Table 1). In 8 cases, the adjacent corresponding non-malignant liver tissue was also acquired for microarray analyses. No patient received preoperative or adjuvant chemo- or radiotherapy.

Samples were resected after appropriate informed consent was obtained and the genetic analysis of the tumor and liver tissue was conducted in accordance with the guidelines of the Declaration of Helsinki (1995) and approved by the local ethic committee. Samples of sufficient weight (> 400 mg) from malignant and corresponding non-malignant liver tissue were excised and snap-frozen in liquid nitrogen within 20 min after excision. Until RNA isolation, samples were stored constantly at -80°C.

Histopathological evaluation

The various architectural patterns and cytological variants of cholangiocarcinoma and especially hepatocellular may complicate the differentiation of primary liver tumors and even that between primary liver tumors and metastases. Therefore, pathological reports with tumor typing, staging (performed using UICC criteria) and grading as well as clinical data were obtained for each tissue sample. Hematoxylin-eosin staining was performed for detection of features like bile canalicular structure and Mallory hyaline bodies. Additional histochemical and immunohistochemical stainings with a panel of antibodies were used routinely (HEP-PAR-1, CK7, CA19-9, CD10, CEA, AFP; Figure 1A and B) in all resected tissue biopsies to confirm the histogenesis of cholangiocellular carcinoma and to exclude hepatocellular carcinoma or metastases. In particular, CK7 is used as an important marker for CCC, as it is significantly overexpressed in CCC compared with HCC and most cases of other adenocarcinoma^[13].

Oligonucleotide array analysis

Sample preparation: Samples were processed with only minor modifications according to the Affymetrix GeneChip® Expression Analysis Manual (Santa Clara, CA/USA). For the extraction of total RNA frozen samples were homogenized in Trizol (Invitrogen, USA). The total RNA yield for each sample was 200-400 µg. After washing with DEPC-treated water (Ambion, USA), total RNA was cleaned using RNEasy mini kits (Quiagen). The amount of total RNA was measured photometrically using a BIO-Photometer (Eppendorf; OD1 E260 = 40 µg/mL).

Preparation of labeled cRNA and hybridization to oligonucleotide arrays: For transcription of total RNA into cDNA, 8 to 10 µg of total RNA was used for cRNA preparation using the SuperScript Choice system (Invitrogen, USA). First-strand cDNA synthesis was primed using a T7-(dT24) oligonucleotide primer

Table 1 Patient demographics and histopathological data

Patient No.	Sex	Age	Histology	Stage	Grading
1	F	57	Adenocarcinoma	pT3pNxpM0	G3
2	F	65	Adenocarcinoma	pT3pN0pM0	G2
3	F	68	Adenocarcinoma	pT2pNxpM0	G2
4	M	74	Adenocarcinoma	pT3pN1pM0	G3
5	F	62	Adenocarcinoma	pT3pN0pM0	G2
6	F	47	Adenocarcinoma	pT3pNxpM0	G2
7	M	73	Adenocarcinoma	pT3pN0pM0	G2
8	F	71	Adenocarcinoma	pT2pNxpM0	G2
9	M	52	Adenocarcinoma	pT3pN2pM0	G2
10	M	65	Adenocarcinoma	pT3pNxpM0	G1

with an RNA polymerase promoter site added to the 3' end. After second-strand synthesis, *in vitro* transcription was performed in the presence of biotin-11-CPT and biotin-16-UTP (Enzo Diagnostics) to produce biotin-labeled cRNA. After fragmentation of the cRNA products (20 µg at 94°C for 35 min) to lengths of 35-200 bp, the samples were added to a hybridization solution to a final cRNA concentration of 0.05 mg/mL. Hybridization was performed by incubation (18-20 h) of 200 µL of the sample to an Affymetrix human GeneChip (Hu 133A) containing 22283 probe sets for known genes/ESTs and stained with streptavidin-phycoerythrin. A Gene Array scanner G2500A (Hewlett Packard, ID) was used to scan the arrays according to procedures described in the Affymetrix manual.

To validate the relative change in gene expression of the most consistently overexpressed gene in all tumor samples (osteopontin, OPN), further analysis with real-time PCR using the LightCycler® system (Roche Diagnostics, Mannheim, Germany) was performed in 8 samples (tumor tissue *vs* corresponding non-malignant liver tissue).

Gene-specific primers corresponding to the coding region were designed using OLIGO software and were obtained from Biomers.net (Ulm, Germany; forward primer OSTEOP: 696 U; GGACAGCCGTGGGAAGG, reverse primer OSTEOP: 810 L; TCAATCACATCGGAATGCTCA). Preliminary experiments were performed to test for the specificity of product formation, determine annealing temperatures, and check for $T_m(\text{Product-Primer Dimer}) > 3^\circ\text{C}$. Validation experiments were performed within a fluorescence signal window excluding primer-dimer formation. The correct PCR efficiency for each target was determined by constructing relative standard curves using five-point half-logarithmic RNA dilutions from one sample. Template concentrations were given arbitrary values of 1, 0.316, 0.1, 0.0316 and 0.01. RT-PCR reactions were performed using the LC RNA Amplification Kit containing SYBR Green I, (Roche Diagnostics; Mannheim, Germany). Amplification was followed by melting curve analysis. Relative values for the initial target concentration in each sample were determined using Light Cycler software 3.5. Crossing points (C_p) were computed using the "2nd derivative maximum method", part of the software above. The relative change in gene expression was computed by pairwise comparison of tumor samples

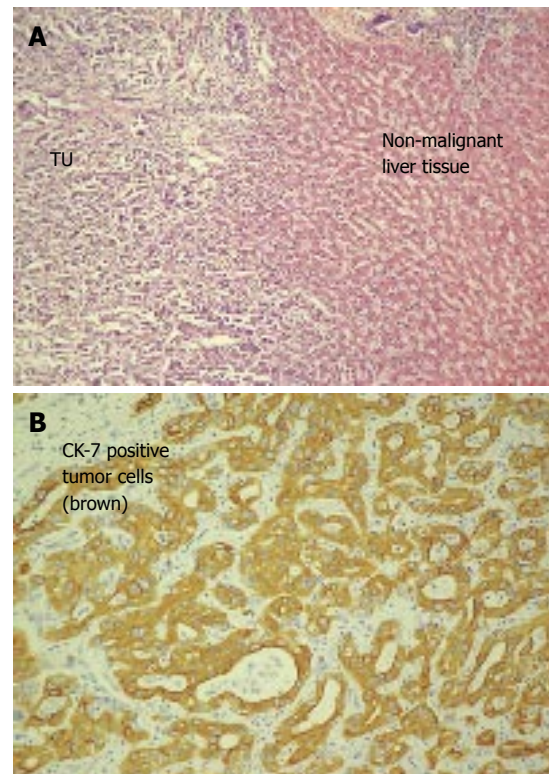


Figure 1 A: HE staining of infiltrative intrahepatic cholangiocarcinoma (Patient No. 6; left tumor tissue with cells of a moderate (G2) differentiated adenocarcinoma, right non-malignant liver tissue); B: Detection of cytokeratin 7 in intrahepatic cholangiocarcinoma (same patient as in A) using immunohistochemistry. Cytokeratin 7 was detected in over 80% of all analyzed CCCs strongly overexpressed (14.6-fold change).

to samples of adjacent normal tissue for each patient.

Statistical analysis

The statistical analyses and presentation of the obtained data were performed in accordance with the MIAME criteria and will be published on the web page (www.matri-gene.de/geneprofiles/CCC).

Preliminary data analysis: Raw data analysis was conducted using the software of the Affymetrix microarray suite (MAS *vs* 5.0.1). MAS produced an expression value plus an index parameter indicating positive or negative detection (present call index) for each of the 22283 probe sets (known genes/ESTs) on the array. Statistical analysis and post-processing were performed using GeneSpring (*vs* 6.1; Silicon Genetics, Redwood City, CA) and Gene-Explore (*vs* 1.1; AppliedMaths, Saint-Martens-Latem, Belgium) software. Mismatch probes acted as specific controls on each array and allowed the direct subtraction of both background and cross-hybridization signals. Only Chip results with scaling factors of 0.5 to 1.8 were accepted for further analyses. Expression values were log2 transformed on the basis of the signal log ratio, given by the comparison of two array results between tumor and non-tumorous tissues.

A *P*-value cut off of < 0.05 (*t*-test) and a fold change difference of ≥ 2 in 70% or more of all analyzed samples were considered to be significant.

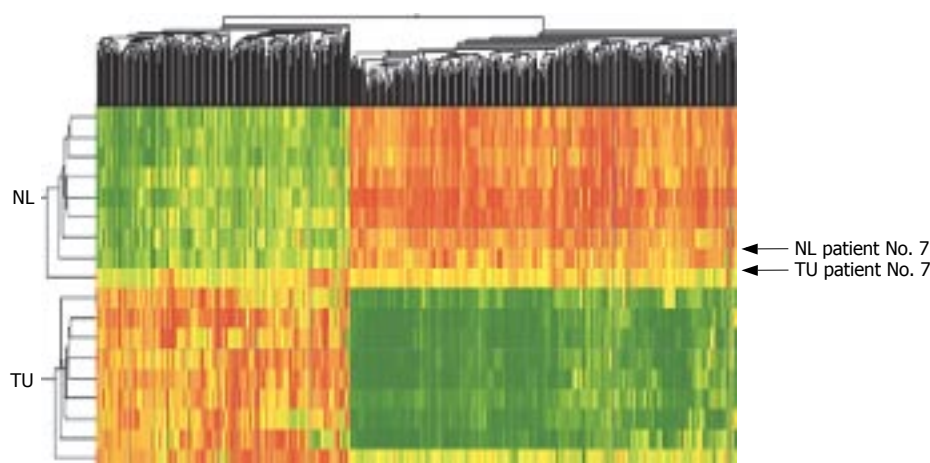


Figure 2 Two-dimensional cluster analysis of CCC (TU) and corresponding non-malignant liver tissues (NL) using 552 dysregulated genes/ESTs (red: overexpressed genes, green: downregulated genes; Pearson's correlation). Dysregulation was defined as different genetic expression in > 70% of all probes with a fold change > 2.0. Horizontal lines show all dysregulated genes in one singular tissue probe, vertical lines show the expression of one gene in all analyzed tumor and non-malignant tissue probes. Using cluster analysis a fast differentiation between tumor tissue and non-malignant tissue was possible in 90% of cases (tumor tissue of Patient No. 7 showed many genetic similarities to non-malignant liver tissue).

Second analysis step - Hierarchical clustering and supervised analysis: To identify differentially expressed genes in CCC, normalized expression data on statistically different genes were used for a paired data analysis by hierarchical clustering with gene trees/experiment trees using either Pearson's correlation or standard correlation algorithms (Genespring *vs* 6.1; GeneExplore *vs* 1.1).

The class predictor program (supplied by GeneSpring 6.1) was used for supervised learning and training (neuronal training) to identify unknown probes as tumor or non-tumorous tissue. A list of the most predictive genes was made for each class and an equal number of genes with the lowest *P*-value using Fischer's exact test were taken from each list. To make a prediction, the class predictor uses the k-nearest-neighbour method.

To detect statistically differentially expressed genes in well and poor differentiated intrahepatic CCC (genetic subclassification), a 1-way ANOVA test was performed. In a second step significantly dysregulated genes ($P < 0.05$) in tumor samples were used for a weighted two-dimensional clustering.

Identification of significantly altered metabolic pathways involved in cholangiocarcinogenesis:

To identify specific signaling or metabolic pathways involved in cholangiocarcinogenesis data on significantly differentially expressed genes were transferred to a public domain software program (GenMAPP 2.0 beta[©]Gladstone Institutes, 2000-2004).

RESULTS

Gene expression profiling of intrahepatic CCC

Primary chip data were screened for RNA quality by 5' to 3' degradation. Of the 22283 probes (genes/ESTs) present on the chip, on average, 43.3% (SD \pm 4.53) and 39.0% (SD \pm 3.48) of genes were expressed in CCC and corresponding non-malignant tissue samples.

Based on the primary data an algorithm was developed to identify and rank the most consistently up- and downregulated genes in CCC compared with non-malignant tissue. All statistically different genes ($P < 0.05$) with a fold change of at least a 2-fold increase or decrease in 70% or higher percentage of the CCC tumor samples

were used to generate a databank of 552 genes/ESTs. Of these, 221 were upregulated and 331 were downregulated. This set of data was used for two-dimensional clustering. Ninety percent of all tumor samples showed a consistent pattern of up- and downregulated genes; only 1 case (No. 7) showed characteristics of both tumor and non-tumor tissue (Figure 2).

To further test the robustness of this cluster, a supervised learning method (SLM) based on neuronal networking was applied. An optimal prediction with subsequent cross-validation was obtained using all significantly dysregulated genes/ESTs and 8 neighbors; using this, all 10 tumor samples could be correctly identified. This resulted in a positive prediction value of 90% ($P < 0.001$) and a negative predictive value of 100% ($P < 0.00023$) of the Gene cluster with the expression pattern of the 552 probes (Table 2).

Using these data, even a specific and fast discrimination between intrahepatic CCC and other malignant liver tumors (HCC, metastases; data not shown), with a high predictive value ($P < 0.001$), was possible by two-dimensional cluster analysis and SLM.

Dysregulated genes and specifically involved metabolic pathways in intrahepatic CCC

CCC gene expression profile data were imported into GeneMapp software to identify specific changes in molecular pathways. Of the 552 dysregulated genes, a total of 364 genes could be assigned to specific metabolic or signaling pathways. Most of the consistently and strongly overexpressed genes were related to cell cycle regulation and DNA replication (15 genes, including *ribonucleoside-diphosphate reductase M2*, *calgizxarin*, *calcyclin*, *BUB1B*) or intracellular signaling (15 genes, including *CD24* and *MARCKS*), genes encoding transcription factors (6 genes, such as *SOX9*), or genes involved in nuclear organization and nucleic metabolism (13 genes, such as *thymidylate synthetase*). Most of the other up-regulated genes could be attributed to gene families, such as genes coding for extracellular matrix and cell adhesion molecules (37 genes, for example, *OPN*, *ADAM9*, *thymosin beta-10*, *integrin alpha-6*), cytoskeleton structure (16 genes, such as *tropomyosin2*, *cytokeratin 7* and *19*) or protein biosynthesis (4 genes).

Table 2 Statistical results obtained using a supervised neuronal training method for discrimination between CCC and non-malignant liver tissue

Condition	True value	Prediction	P value
Pat. No. 1, non-malignant	Normal liver tissue	Non-malignant	0.00023
Pat. No. 2, non-malignant	Normal liver tissue	Non-malignant	0.00023
Pat. No. 3, non-malignant	Normal liver tissue	Non-malignant	0.00023
Pat. No. 4, non-malignant	Normal liver tissue	Non-malignant	0.00023
Pat. No. 5, non-malignant	Normal liver tissue	Non-malignant	0.00023
Pat. No. 6, non-malignant	Normal liver tissue	Non-malignant	0.00023
Pat. No. 7, non-malignant	Normal liver tissue	Non-malignant	0.00023
Pat. No. 8, non-malignant	Normal liver tissue	Non-malignant	0.00023
Pat. No. 1, CCC	CCC	Malignant	0.00103
Pat. No. 2, CCC	CCC	Malignant	0.00103
Pat. No. 3, CCC	CCC	Malignant	0.00103
Pat. No. 4, CCC	CCC	Malignant	0.00103
Pat. No. 5, CCC	CCC	Malignant	0.00103
Pat. No. 6, CCC	CCC	Malignant	0.00103
Pat. No. 7, CCC	CCC	Malignant	0.16100
Pat. No. 8, CCC	CCC	Malignant	0.00103
Pat. No. 9, CCC	CCC	Malignant	0.00103
Pat. No. 10, CCC	CCC	Malignant	0.00103

Statistical results of the ability to differentiate between normal and malignant liver tissue (CCC) using a supervised neuronal training method. In all cases, a fast and correct differentiation was possible with a high positive ($P < 0.001$ in 90%) and negative (100%, $P < 0.00023$) predictive value.

According to an upregulation of genes involved in cell cycle regulation, most of the genes encoding proteins involved in cellular apoptosis (7 genes, such as growth-arrest specific protein 2, *CIDE-B*) were found to be down-regulated in intrahepatic CCC. Furthermore, a significant suppression of genes encoding proteins important for metabolic pathways like amino acid metabolism (39 genes), carbohydrate and fat metabolism (13 and 11 genes) or electron transport (27 genes) was noted. For clarity, only the 41 most consistently dysregulated (increased or decreased) genes ($P < 0.001$; 4-fold change in 100%) are listed in Table 3.

Separation of well and poorly differentiated intrahepatic CCC by 2-dimensional cluster analysis

For subclassification of intrahepatic CCC in relation to histopathological differentiation (grading) a weighted two-dimensional clustering of the tumor samples was performed. All expressed genes/ESTs in the tumor samples (43.3%, approx. 9650 genes/ESTs) were grouped according to the histological grading ranging from 1 to 3 and then ranked according to their significantly differential expression values. Differentiation of the gene expression profiles of tumor samples, according to the histopathological grading of 1 and 3, identified a total of 136 dysregulated genes ($P < 0.05$ and two-fold change upregulation or downregulation in ≥ 70), which were used for a weighted two-dimensional cluster analysis (Figure 3). Interestingly, upregulation of specific cell surface antigens such as cytokeratins (for example, cytokeratin 6, 7, 13 and 15) and specific membrane proteins (such as EMP1, EVA1 and proteoglycan 2) was detected in well differentiated tumor samples (G1, G2) when compared with more dedifferentiated tumor samples. In contrast, G3-tumor samples showed a relative overexpression of genes

involved in G-protein signaling and genes involved in transcription (Table 4).

Results of RT-PCR for detection of dysregulated OPN expression

The most consistently overexpressed gene (median fold change 33.5) in all analyzed intrahepatic CCC samples was that encoding the glycoprotein OPN.

To validate this observation the expression levels of OPN were confirmed by quantitative RT-PCR in 16 tissue samples (malignant and corresponding non-malignant tissue from 8 patients) using the LightCycler® system. In general, the changes in OPN expression levels detected by microarray reflected the results obtained by RT-PCR. However, the dynamic range of real-time PCR results was greater than that of the microarray data (Figure 4).

DISCUSSION

The poor response of cholangiocarcinoma to therapy, along with the concerning worldwide increase in morbidity and mortality^[4,5], highlights the need for increased efforts to understand the etiology and pathogenesis of this primary liver cancer.

Previous studies have examined the potential role of known genes involved in human carcinogenesis to elucidate the molecular pathways involved in cholangiocellular carcinoma, for example, members of the type I family of growth factor receptors (EGF-R, c-erbB-2)^[14-16], (proto-) oncogenes (c-met, c-Ki-ras, Cyclin D)^[17-19], dysregulated tumor-suppressor genes (p53, pRB, p16INK4a, p21^{WAF1})^[20,21], and apoptosis-related genes (bcl2, FAS-L, BAX)^[22,23]. Other studies have shown microsatellite instability and loss of heterogeneity (LOH) as contributing factors in the development of CCC^[24,25].

Excepting the actual data there are only two other reports using gene expression analysis to detect dysregulated genes and prognostic markers in cholangiocarcinogenesis^[26,27]. The present study identified a high number of differentially expressed genes and ESTs in CCC (43.6 vs 39.01%, $P > 0.05$). Based on a relatively conservative arbitrarily chosen algorithm, a gene expression profile of 552 dysregulated genes (221 genes upregulated, 331 genes downregulated) for CCC was generated. This profile was validated based on a neuronal training method by its ability to differentiate the CCC profile from the expression profiles of corresponding and non-corresponding non-malignant liver tissues. This method achieved a fast and reproducible differentiation between CCC and non-malignant liver tissue in all samples, as well as between CCC and other malignant tissues (HCC, liver metastases; data not shown). One tumor sample (No. 7) also showed characteristics of non-malignant liver tissue in that the tissue expressed a number of genes that were more frequently expressed in non-diseased human liver than in CCC samples. The sample was attributed to the non-malignant tissue group in two-dimensional hierarchical clustering. A potential reason for this could be a putative contamination of tumor sample with normal liver tissue, especially owing to the fact that no microdissection of tumor tissue was used. However, using a supervised neuronal training method to

Table 3 Most significantly dysregulated genes (> 4-fold change in 100% of all cases) in intrahepatic cholangiocarcinoma - relation to known cellular functions and metabolic pathways (using the GenMAPP® software)

Swiss prot	GenMAPP ID	Gene ID	Dysregulated in %	Name	fc inc (median)	fc dec (median)
Cell adhesion (cell-cell/cell-matrix)-extracellular matrix						
OSTP_HUMAN	P10451	SPP1	100	Osteopontin precursor (Bone sialoprotein 1/SPP-1 1)	33.5	
Q13443	Q13443	ADAM9	100	A disintegrin and metalloproteinase domain 9 (meltrin γ)	10.4	
TYBO_HUMAN	P13472	TMSB10	100	Thymosin beta-10	5.3	
ITA6_HUMAN	P23229	ITGA6	100	Integrin alpha-6 precursor	4.5	
Signal transduction - G protein signaling						
CD24_HUMAN	P25063	CD24	100	Signal transducer CD24 precursor	11.8	
IQG1_HUMAN	P46940	IQGAP1	100	Ras GTPase-activating-like protein IQGAP1	9.1	
ECT2_HUMAN	Q9H8V3	ECT2	100	ECT2 protein (Epithelial cell transforming 2 oncogene)	6.3	
MACS_HUMAN	P29966	MACS	100	Myristolated alanine-rich C-kinase substrate (MARCKS)	4.6	
Q9BUV5	Q9BUV5	INSIG1	100	Similar to insulin induced gene 1		5
Cell cycle - DNA replication						
RIR2_HUMAN	P31350	RRM2	100	Ribonucleoside-diphosphate reductase M2 chain	16.2	
S111_HUMAN	P31949	S100A11	100	Calgizzarin (S100C protein)	15.5	
O00496	O00496	IPL	100	Tumor suppressing subtransferable candidate 3	14.1	
S106_HUMAN	P06703	S100A6	100	Calcyclin (Growth factor inducible protein 2A9)	6.9	
Transcription						
SOX9_HUMAN	P48436	SOX9	100	Transcription factor SOX9	4.1	
NRI3_HUMAN	Q14994	NR1/3	100	Orphan nuclear receptor NR1/3		6.8
Cell motility - cytoskeleton						
SPT2_HUMAN	O53291	SPINT2	100	Kunitz-type protease inhibitor 2 precursor (Hepatocyte growth factor activator inhibitor type 2)	5.8	
Amino acid metabolism						
BHMT_HUMAN	Q93088	BHMT	100	Betaine-homocysteine S-methyltransferase		48.5
METL_HUMAN	Q00266	MAT1A	100	S-adenosylmethionine synthetase alpha and beta forms		38.5
ATTY_HUMAN	P17735	TAT	100	Tyrosine aminotransferase		27.0
SPYA_HUMAN	P21549	AGXT	100	Serine-pyruvate aminotransferase		22.0
HUTH_HUMAN	P42357	HAL	100	Histidine ammonia-lyase		16.7
CGL_HUMAN	P32929	CTH	100	Cystathione gamma-lyase (Gamma-Cystathionase)		9.9
GLSL_HUMAN	Q9UI32	GA	100	Glutaminase, liver isoform, mitochondrial precursor		9.5
GAMT_HUMAN	Q14353	GAMT	100	Guanidinoacetate N-methyltransferase		8.4
SDHL_HUMAN	P20132	SDS	100	L-serine dehydratase		7.1
Carbohydrate/monosaccharide metabolism/catabolism						
KPY2_HUMAN	P14786	PKM2	100	Pyruvate kinase, M2 isozyme	26.7	
ALFA_HUMAN	P04075	ALDOA	100	Fructose biphosphate aldolase A (Lung cancer antigen)	16.3	
PPCC_HUMAN	Q16822	PCK1	100	Phosphoenolpyruvate carboxykinase, cytosolic		16.7
Q9H277	Q9H277	Q9H227	100	Cytosolic beta-glucosidase		7.6
Electron transport						
ACDB_HUMAN	P45954	ACADSB	100	Acyl-CoA dehydrogenase, branched chain specific		14.1
CPA6_HUMAN	X13929	CYP2A6	100	Cytochrome P450 2A6		11.9
CP12_HUMAN	P05177	CYP1A2	100	Cytochrome P450 1A2		10.0
Protein metabolism/proteolysis						
BAE2_HUMAN	Q9Y5Z0	BACE2	100	Beta secretase 2 precursor	10.5	
Q9UJ28	Q9UJ28	GALNT7	100	UDP-GalNAc: N-acetylgalactosaminyltransferase 7	5.4	
CATC_HUMAN	P53634	CTSC	100	Dipeptidyl-peptidase I precursor	4.7	
GLMT_HUMAN	Q14749	GNMT	100	Glycine N-methyltransferase		49.5
Transport						
CHLR_HUMAN	P17516	AKR1C4	100	Chlordecone reductase		22.0
APF_HUMAN	Q13790	APOF	100	Apolipoprotein F precursor		15.5
Lipid binding/metabolism						
Q9Y2P5	Q9Y2P5	Q9Y2P5	100	Very-long-chain AcylCoA synthetase homolog 2		23.7
VLCS_HUMAN	O14975	FACVL1	100	Very-long-chain AcylCoA synthetase		16.6
MMSA_HUMAN	Q02252	MMSDH	100	Methylmalonate-semialdehyde dehydrogenase		8.1

reanalyze all of the obtained array data, correct assignment to malignant and non-malignant tissue groups was possible for all samples (including No. 7).

Previous studies have shown a correlation between differentially expressed genes and tumor progression or tumor dedifferentiation in various human tumors^[28,29]. In the present study we used two-dimensional cluster

analysis with all expressed genes in the tumor samples (> 9000 genes/ESTs) for the detection of statistically significantly differentially expressed genes in relation to tumor differentiation (grading). This method detected 136 differentially expressed genes when tumor samples with low (G1) and high (G3) grading were compared. Because of the small number of analyzed tumor

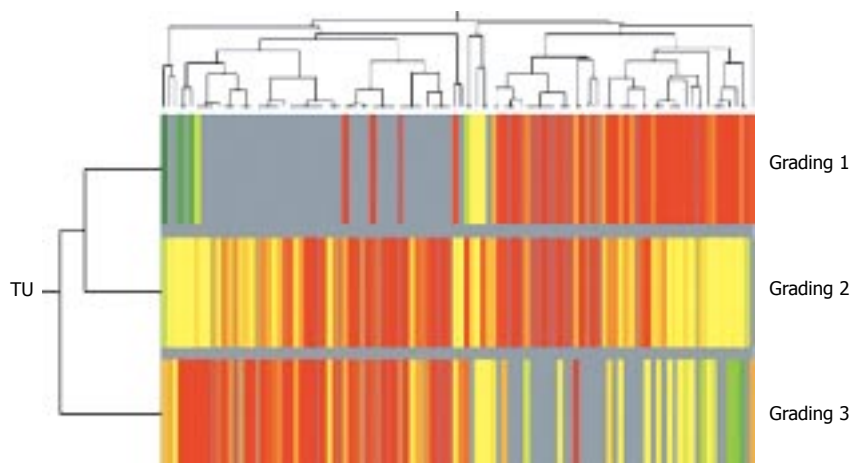


Figure 3 Subclassification of intrahepatic CCC using two-dimensional cluster analysis of 136 different regulated genes/ESTs (dysregulation in more than 70% of all probes, fold change of genetic expression > 2.0; Pearson's correlation) in relation to histopathological findings (well to poor differentiated tumor tissue). Horizontal lines show all dysregulated genes in one singular tumor tissue probe, vertical lines show the expression of one gene in all analyzed G1-, G2- and G3-tumors. Overexpressed genes are coloured red, downregulated genes are shown in green).

Table 4 Expression levels of dysregulated genes in intrahepatic cholangiocarcinoma in relation to tumor differentiation according histopathological criteria (grading 1 to 3)

Probe set	Grading 1	Grading 2	Grading 3	Gene function
204857_at	3.09	4.14 (2.9-5.8)	No data	Hs. MAD1 (mitotic arrest deficient, yeast, homolog)-like 1
211571_s_at	3.49	0.98 (0.7-1.3)	1.411	mRNA for proteoglycan PG-M (V3), proteoglycan 2
209016_s_at	3.93	1.25 (0.3-6.7)	0.96 (0.92-1.0)	Hs. similar to keratin 7 (KRT7)
201325_s_at	4.27	4.5 (3.0-6.7)	1.08	Hs. epithelial membrane protein 1 (EMP1), mRNA
203779_s_at	5	No data	No data	Hs. epithelial V-like antigen 1 (EVA1), mRNA
211796	7.82	1.53 (1.1-1.9)	1.16	Hs. T cell receptor beta chain (TCRBV13S1-TCRBJ2S1) mRNA
204734_at	7.97	8.5 (6.3-11.4)	No data	Hs. keratin 15 (KRT15), mRNA
209126_x_at	10.26	8.254	No data	Hs. keratin 6 isoform K6f (KRT6F) mRNA
207935_s_at	19.94	10.4	No data	Hs. keratin 13 (KRT13), mRNA
217109_at	22.75	No data	No data	Hs. partial mRNA for sv7-MUC4 apomucin
205157_s_at	34.79	10.36	No data	Hs. keratin 17 (KRT17), mRNA
209070_s_at	No data	3.188	7.54 (6.9-8.2)	Hs. regulator of G-protein signaling 5, mRNA
204338_s_at	No data	18.98	128.5	Hs. regulator of G-protein signaling 4 (RGS4), mRNA
203638_s_at	No data	1.14 (0.7-2.3)	4.16 (2.7-6.4)	Hs. FGF receptor 2, keratinocyte growth factor receptor
208343_s_at	No data	1.36 (1.1-1.8)	2.45 (1.2-4.9)	Hs. CYP7A promoter binding factor mRNA, nuclear receptor 5
207846_at	No data	No data	1.414	Hs. POU domain, class 1, transcription factor 1 (Pit1)

No data: No significant (< 2-fold change) or consistent (< 70% of all cases) change in the expression level of the gene in this subgroup of CCC (G1-, G2- or G3-tumors).

samples (one highly differentiated tumor (G1) among 10 samples) it was not possible to create an unambiguous clear correlation between all dysregulated genes and the histopathologic grading. However, an obvious trend towards a higher expression of specific cell surface proteins (EMP1, EVA1, proteoglycan2) and intermediate filaments (cytokeratin 6, 7, 13, 15, 17) in well-differentiated tumors was observed, whereas samples of high-grade intrahepatic CCC had an elevated expression of genes involved in G-protein signaling and nuclear transcription. Pathological alterations to the chemical composition, molecular structure, or spatial arrangement of the liver matrix will ultimately lead to specific changes in the intermediate filament pattern in human cholangiocytes. It can be assumed that the dedifferentiation of tumor cells in CCC results in pathological alterations of cell surface antigens, cell-to-cell and cell-to-matrix attachment, followed by a switch from physiological to pathological cell-activation. More investigations are needed to elucidate the role of dysregulated genes in tumor progression with histomorphological tumor dedifferentiation in cholangiocarcinogenesis.

To further validate the microarray data, real-time PCR

was performed. The gene encoding OPN was identified as the highest and most consistently overexpressed gene (33.5-fold change) in all analyzed tumor samples. The expression profile determined by real-time PCR correlated well with the resulting ratios carried out with the gene expression data of the analyzed CCC. However, as has been shown previously for other similar comparisons^[30,31], the dynamic range of real-time PCR was about 1.5-fold to 10-fold higher than that of array analysis.

Using oligonucleotide arrays we were able to identify genes already known to be dysregulated in other human cancers^[32,33]. However, a significant number of the genes identified to show a significant alteration in their expression values, have not been known to be involved in cholangiocarcinogenesis until now. By incorporating the expression data into GenMAPP software, a specific pattern of signaling and metabolic pathways as well as changes in cell cycle regulators specific for CCC could be generated. For example, changes in apoptosis-regulating genes consistent with the decreased cell death rate typical of tumor cells were identified. Generally, the changes in cell cycle metabolism are well in line with a pattern of increased cell growth. However, the major focus of this

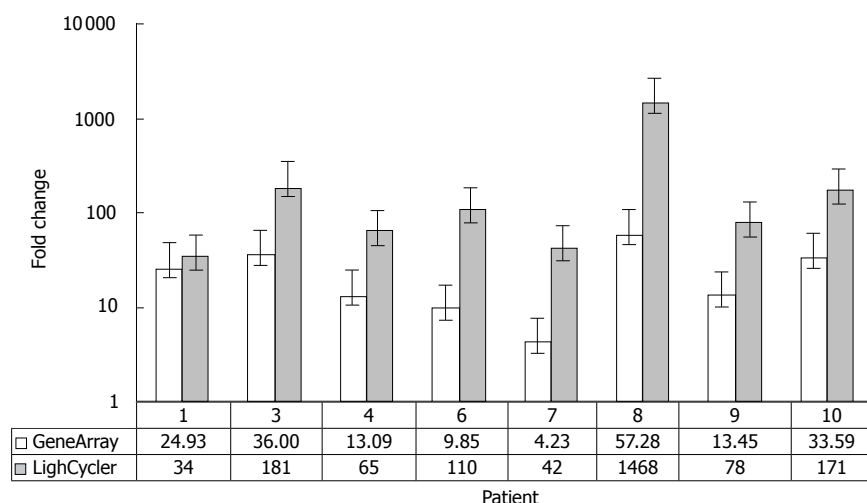


Figure 4 Comparison between expression values of osteopontin in 8 intrahepatic CCCs estimated by RT-PCR (LightCycler®System; grey) and gene expression data (HU 133A, Affymetrix; white). The detected changes in osteopontin expression levels measured by RT-PCR reflected very well the changes in gene expression between tumor and non-malignant liver tissue obtained by microarray analysis. The results of RT-PCR revealed larger changes than the microarray data in all cases.

study was to establish a specific genetic profile of CCC for differentiation against non-malignant liver tissue and other malignant liver tumors (HCC, metastases) for clinical routine. This approach intentionally did not take into consideration the different origins of the analyzed tissue probes (tumorous epithelium cells *vs* non-malignant transformed hepatocytes). Thus, it remains a possibility that our observations of the downregulation of genes involved in specific metabolic pathways, such as amino acid metabolism, may be a consequence of the different origin of malignant and non-malignant tissue rather than a specific feature of cholangiocarcinogenesis. Nevertheless, our own unpublished data revealed similarities between the genetic profiles of other malignant liver tumors (HCC, metastases of adenocarcinomas) and CCC with a downregulation of hepatocyte-specific metabolic functions and an upregulation of genes well known to be involved in human carcinogenesis.

The gene encoding OPN, a secreted adhesive glycoprotein was identified as the most consistently overexpressed gene in all CCC samples (see above). OPN has been shown to be overexpressed in excessive amounts in a variety of human carcinomas and its expression level has been linked to the recurrence of metastasis or to clinical progression^[34-36].

OPN is not a typical oncogene; it is mutationally activated in cancer cells, but it is regulated by a variety of stimuli, such as the Wnt/Tcf signaling pathway, steroid receptors, growth factors, TNF-alpha and transcription factors, such as AP-1 and Ras proto-oncogene^[37]. OPN is a ligand of CD44, binds to alphaV-containing integrins and has an important role in malignant cell attachment and tumor invasion^[38,39]. Potential mechanisms of OPN in human carcinogenesis include the regulation of different signaling cascades through activation of various kinases^[40]. Thus, OPN seems to be a potent anti-apoptotic factor *via* inhibition of caspase 3 activation^[41]; it also induces increased activation of promatrix metalloproteinase-2 (pro-MMP2, mediated by nuclear factor kappaB) and secretion of urokinase-type plasminogen activator^[42].

Using gene expression profiling, OPN has been shown to be one of the highest overexpressed genes in hepatocellular carcinoma (HCC) and it has been shown

the expression of OPN correlates with earlier recurrence, poorer prognosis and metastasis in HCC^[43,44]. To the best of our knowledge, there is only one report on OPN in human cholangiocarcinogenesis^[45]. Interestingly, Terashi *et al* observed a correlation between low OPN levels and tumor aggressiveness, whereas another group found high levels of the glycoprotein in CCC in a rat model system^[46]. Therefore, it remains to be shown whether OPN is a potential prognostic marker and target for anticancer treatment in CCC.

In conclusion, the present study represents one of the first attempts to establish a gene expression pattern of intrahepatic CCC by genetic comprehensive analysis. Based on the analysis of a subgroup of 364 genes, a specific pattern of changes in metabolic and signaling pathways, as well as receptors, cell cycle changes and alterations in apoptosis could be identified. The observed changes are consistent with some of the known characteristics of CCCs in particular and tumors in general. The gene expression profile appears to be useful as a diagnostic tool, especially in terms of differentiation from other liver masses as well as for the subclassification of intrahepatic CCC in comparison to histopathological findings.

Conclusively, besides a number of known overexpressed tumor-related genes in intrahepatic CCC, we describe here for the first time other strongly and consistently dysregulated genes in cholangiocarcinogenesis, which are well known to be involved in other human cancers. Although our data at present do not support a correlation between the expression of these genes and tumor stage for CCC, this gene expression pattern is in line with the usually observed bad clinical prognosis of this cancer. Another evolving application based on the profile described in this report and the observation of OPN as the most consistently overexpressed gene might be the identification of novel therapeutic targets and related genes predicting survival and outcomes of therapeutic modalities.

COMMENTS

Background

Cholangiocarcinoma (CCC) is the second most common primary hepatic malignancy with increasing incidence in western countries. Despite recent progress

in various imaging modalities, intrahepatic CCC is in most cases advanced by the time of diagnosis. Aggravating circumstances leading to a poor prognosis with median survival times of only three to six months are the limited curative treatment options by surgical resection and the low effect of palliative chemotherapy in CCC. Therefore a better understanding of human cholangiocarcinogenesis is strongly needed to establish new diagnostic markers and therapeutic approaches.

Research frontiers

Despite the advances in genomic profiling of many human malignancies in recent years, there have only been a few reports of gene expression analysis in CCC. In this study, oligonucleotide arrays were used to detect differentially expressed genes and to establish a specific genetic profile of intrahepatic CCC. To subclassify between well and poorly differentiated tumors, additional analyses were performed.

Innovations and breakthroughs

A total of 41 genes that were strongly deregulated (upregulated or downregulated in 100%, $fc > 4$) in CCC were detected by gene expression analysis. For the first time, a subclassification of intrahepatic CCC in relation to malignant differentiation using oligonucleotide arrays was performed. The gene encoding osteopontin (OPN) was the most overexpressed gene with a high fold-change of 33.5, which was confirmed by RT-PCR.

Applications

Besides a number of known overexpressed tumor-related genes in intrahepatic CCC, other strong and consistently dysregulated genes were described for the first time in human cholangiocarcinogenesis. Another evolving application based on the profile described in this report and the observation of OPN as the most consistently overexpressed gene might be the identification of novel therapeutic targets and related genes predicting survival and outcomes of therapeutic modalities.

Peer review

The manuscript by Hass *et al* describes a microarray analysis of genetic changes in human cholangiocarcinoma samples compared to non-tumor tissue. The analysis revealed very intriguing data with respect to the overexpression of a number of genes, the most consistent of which is OPN, and further analysis with respect to tumor grade was performed.

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Anti-inflammatory activity of probiotic *Bifidobacterium*: Enhancement of IL-10 production in peripheral blood mononuclear cells from ulcerative colitis patients and inhibition of IL-8 secretion in HT-29 cells

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Abstract

AIM: To determine the anti-inflammatory activity of probiotic *Bifidobacteria* in *Bifidobacteria*-fermented milk (BFM) which is effective against active ulcerative colitis (UC) and exacerbations of UC, and to explore the immunoregulatory mechanisms.

METHODS: Peripheral blood mononuclear cells (PBMNC) from UC patients or HT-29 cells were co-cultured with heat-killed probiotic bacteria or culture supernatant of *Bifidobacterium breve* strain Yakult (BbrY) or *Bifidobacterium bifidum* strain Yakult (BbiY) to estimate the amount of IL-10 or IL-8 secreted.

RESULTS: Both strains of probiotic *Bifidobacteria* contained in the BFM induced IL-10 production in PBMNC from UC patients, though BbrY was more effective than BbiY. Conditioned medium (CM) and DNA of both strains inhibited IL-8 secretion in HT-29 cells stimulated with TNF- α , whereas no such effect was observed with heat-killed bacteria. The inhibitory effect of CM derived from BbiY was greater than that of CM derived from BbrY. DNAs of the two strains had a comparable inhibitory activity against the secretion of IL-8. CM of BbiY induced a repression of IL-8 gene expression with a higher expression of I κ B- ζ mRNA 4 h after culture of HT-29 cells compared to that in the absence of CM.

CONCLUSION: Probiotic *Bifidobacterium* strains in BFM enhance IL-10 production in PBMNC and inhibit IL-8 secretion in intestinal epithelial cells, suggesting that BFM has anti-inflammatory effects against ulcerative colitis.

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Key words: *Bifidobacterium*; Ulcerative colitis; Anti-inflammatory; Interleukin-10; Interleukin-8

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INTRODUCTION

Ulcerative colitis (UC) is assumed to be a result of an impaired intestinal immune response to intestinal environmental antigens such as ubiquitous microflora^[1,2]. There is evidence that probiotic treatment is effective against UC^[3-6]. We have previously shown that treatment with *bifidobacteria*-fermented milk (BFM) containing live *bifidobacteria* is more effective than the usual treatment in inducing^[7] and maintaining remission^[8] of ulcerative colitis. An improvement in the composition of intestinal flora by BFM may prevent the overgrowth of potentially pathogenic bacteria such as *Bacteroides vulgatus*, but we hypothesized that probiotic strains of *bifidobacteria* in BFM may directly interfere with the host signaling events that drive the intestinal inflammatory response. In inflammatory bowel disease, IL-10 is a cytokine of particular therapeutic interest since it has been shown in animal models that interleukin (IL)-10(-/-) mice spontane-

ously develop intestinal inflammation. IL-10 plays a key role in the control of inflammatory responses to enteric organisms^[9-12]. More recently, it has been shown in animal models that probiotic strains displaying an *in vitro* potential to induce higher levels of the anti-inflammatory cytokine IL-10 and lower levels of the inflammatory cytokine IL-12, offer the best protection against *in vivo* colitis in the model^[13]. A genetically engineered *Lactococcus lactis* *thy12* producing IL-10 ameliorated colitis in two models of experimental colitis, providing proof of principal that topically delivers IL-10, can be therapeutically efficacious^[14] and a recent proof-of-principle experiment using this transgenic bacterium expressing IL-10 in 10 patients with Crohn's disease showed efficacy^[15]. In addition, there is an increasing amount of evidence to suggest that the potent neutrophil chemoattractant, IL-8, has an important role in the pathogenesis of inflammatory bowel disease (IBD)^[16-19]. Recently, a higher concentration of IL-8 was found in more histologically inflamed tissue segments from pediatric IBD patients, suggesting that IL-8-specific therapies may universally modify the inflammatory activity in IBD patients^[20]. In this study, we focused on the effect of probiotic strains on the secretion of IL-10 by peripheral blood mononuclear cells and also the production of IL-8 by intestinal epithelial cells.

MATERIALS AND METHODS

Bacteria and related preparations

Bifidobacterium bifidum strain Yakult (BbiY) and *Bifidobacterium breve* strain Yakult (BbrY) were grown in MRS broth (Becton, Dickinson and Company, Sparks, MD). Heat-killed BbiY or BbrY was prepared by heating bacteria resuspended in distilled water at 100°C for 30 min, and then lyophilized^[21]. For the preparation of conditioned medium (CM), bacteria grown in MRS broth were collected by centrifugation and cultured over 16 h in RPMI-1640 medium (Sigma-Aldrich, St Louis, MO) containing 10% fetal calf serum (FCS) and 2% lactose, then centrifuged^[22]. The supernatant was filtrated on a 0.22 µm membrane and neutralized with sodium hydroxide. To characterize the active component in CM, it was separated into fractions of more than and less than 3 kDa through Centricon YM-3 (Millipore, Bedford, MA), adjusted to the initial volume, and then to heat treatment at 100°C for 15 min^[23]. DNA was isolated using the method of Yuki^[24] with a slight modification. Briefly, bacterial cells were suspended in Tris-EDTA buffer (pH 8.0) containing 0.5 mol/L sucrose and treated with N-acetylmuramidase SG (Seikagaku Corp., Tokyo, Japan) and lysozyme (Sigma-Aldrich) at 37°C for 1 h. The cells were lysed by addition of sodium dodecyl sulfate and proteinase K (Sigma-Aldrich) followed by a 60-min incubation at 65°C. Deproteinization was done by extraction with Tris-saturated phenol and phenol/chloroform/isoamyl alcohol (25:24:1). Finally, DNA was precipitated by ethanol.

Peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMNC) were isolated from peripheral blood of UC patients by Ficoll-Conray (Lymphosepar I; Immuno-Biological Laboratories, Takasaki, Japan) density gradient centrifugation. Table 1

summarizes the patient characteristics. All 9 patients (outpatient) had active UC which was moderate or mild (1 mild, 8 moderate) according to the criteria of Truelove & Witts^[25]. All patients received a standard therapeutic regimen consisting of oral 5-ASA (mesalazine) and five of the 9 patients with active UC took a low dose of oral predonine. Cells (2×10^5) were cultured with heat-killed bacteria (10 µg/mL) in 200 µL of AIM-V medium (Invitrogen Corp., Carlsbad, CA) in a flat-bottomed 96-well culture plate (Nunc, Roskilde, Denmark) for 48 h. Supernatant was collected and frozen until cytokine levels were quantified. In each assay, a positive control with lipopolysaccharide (LPS, 10 µg/mL) added to PBMNC and a negative control with no stimuli were included.

Quantification of cytokine levels in culture supernatant

IL-10 and IL-8 levels in the culture supernatant were determined by sandwich enzyme-linked immunosorbent assay (ELISA). IL-10 was detected using anti-human IL-10 antibody (51-26171E, BD Biosciences PharMingen, Franklin Lakes, NJ) and biotinylated antibody (51-26172E, BD Biosciences PharMingen). IL-8 was detected using anti-human IL-8 antibody (AHC0932, Invitrogen BioSource) and biotinylated anti-human IL-8 antibody (AHC0789, Invitrogen BioSource).

HT-29 cell culture

HT-29 cells were cultured at 37°C in an atmosphere containing 50 mL/L CO₂ in RPMI-1640 medium containing 10% FCS, 1 mmol/L sodium pyruvate (Invitrogen) and 10 mmol/L 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES). Confluent monolayers were incubated in 96-well or 24-well tissue culture plates with human TNF-α (10 ng/mL, PeproTech, London, UK).

Quantitative RT-PCR

Total RNA was isolated from HT-29 cells using the TRIzol reagent (Invitrogen) according to the manufacturer's instructions. The RNA was reverse transcribed into cDNA using a SuperScript First-Strand Synthesis System kit (Invitrogen). Real-time quantitative PCR was performed in an ABI prism 7500 Real Time PCR System (Applied Biosystems, Foster City, CA) with the SYBR Premix Ex Taq (Takara Bio, Shiga, Japan). The following primers were used to amplify cDNA fragments: IL-8: forward: 5'-ACACTGCGCCAAACACAGAAATTA-3', reverse: 5'-TTTGCTTGAAGTTTCACTGGCATC-3'; GAPDH: forward: 5'-GCACCGTCAAGGCTGAGAAC-3', reverse: 5'-ATGGTGCTGAAGACGCCAGT-3'; IκB-ζ: forward: 5'-ACGCGCAAACATGAGTCCAG-3', reverse: 5'-CTCAGCAGCAGCAACAGCATC-3'. All results were finally determined after correction with GAPDH expression.

Statistical analysis

Results were expressed as mean ± SD. Differences in mean values between groups were analyzed with Student's *t*-test.

RESULTS

Effect of probiotic bacteria on IL-10 secretion in PBMNC

The secretion of IL-10 increased in all the cultures of

Table 1 Characteristics of the patients with ulcerative colitis

Patient No.	Age	Sex	Disease extent	Clinical pattern	Disease duration (yr)	Disease severity	Treatment		
							5-ASA (mesalazine)	(SASP)	Steroid (predonine)
1	23	Male	Total	Relapsing	6	Moderate	+		
2	38	Female	Total	Relapsing	2	Moderate	+		+
3	32	Male	Total	Relapsing	2	Moderate	+		+
4	36	Female	Proctitis	Relapsing	15	Mild		+	
5	23	Male	Total	First	3	Moderate	+		
6	20	Male	Total	Relapsing	2	Moderate	+		+
7	25	Male	Total	First	0.5	Moderate	+		
8	42	Male	Left-sided	Chronic	7	Moderate	+		+
9	36	Male	Left-sided	Relapsing	14	Moderate	+		+

SASP: Salazosulphapyridine.

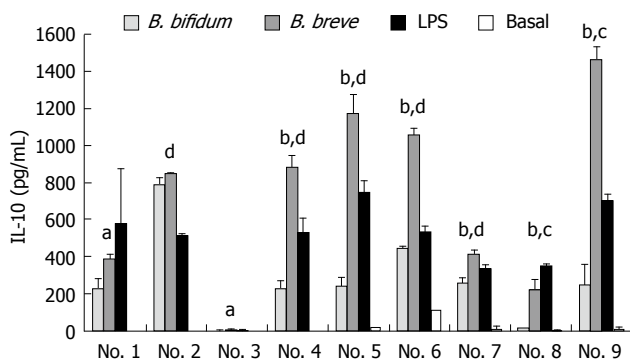


Figure 1 Effects of probiotic bifidobacteria on IL-10 production in PBMC. PBMC were isolated from 9 ulcerative-colitis patients and incubated with heat-killed probiotic BbiY or BbrY (10 μ g/mL), or LPS. At forty-eight hours after incubation, the IL-10 concentration was determined by ELISA (mean \pm SD, $n = 3$). ^a $P < 0.05$, ^b $P < 0.01$ vs BbiY; ^c $P < 0.05$, ^d $P < 0.01$ vs LPS.

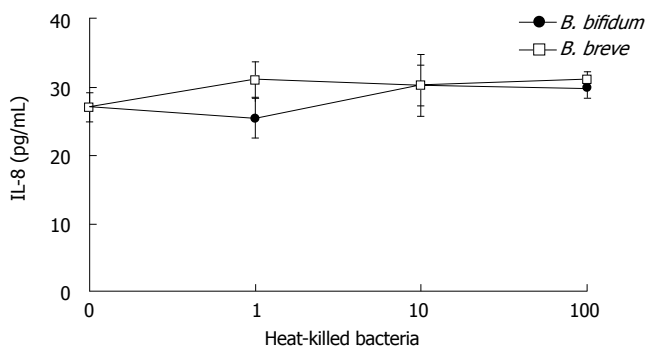


Figure 2 Effects of probiotic bifidobacteria on IL-8 production in HT-29 cells. HT-29 cells were stimulated with TNF- α (10 ng/mL) in the absence or presence of various concentrations of probiotic bacteria. Six hours after incubation, the IL-8 concentration was determined by ELISA (mean \pm SD, $n = 4$).

PBMC isolated from the nine UC patients with a historical record of the treatment as shown in Table 1 in the presence of heat-killed BbrY or BbiY, compared to the basal secretion in the absence of probiotic bacteria (Figure 1). BbrY induced significantly higher levels of IL-10 than BbiY in 8 out of the 9 PBMC preparations. Incubation with BbrY also elicited significantly higher levels of IL-10 than that with LPS (10 μ g/mL) in 6 out of the 9 PBMC preparations.

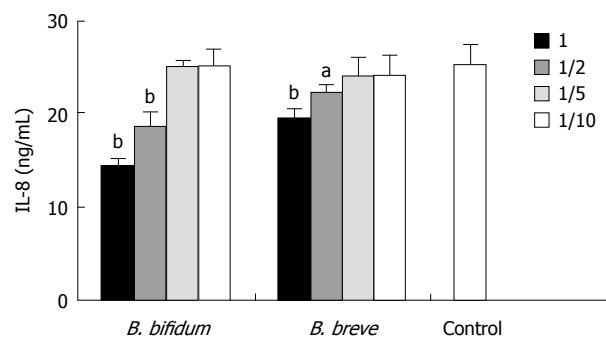


Figure 3 Effects of probiotic bifidobacteria-derived CMs on IL-8 production in HT-29 cells. HT-29 cells were stimulated with TNF- α in the absence or presence of graded dilutions of CMs. CMs were prepared as described in the Methods. Other experimental conditions are shown in Figure 2. ^a $P < 0.05$, ^b $P < 0.01$ vs control.

Effect of probiotic Bifidobacterium on IL-8 secretion in TNF- α -stimulated HT-29 cells

HT-29 cells were incubated with TNF- α for six hours in the presence or absence of heat-killed BbiY and BbrY. Neither of the heat-killed probiotic bacteria had an effect on the secretion of IL-8 at the concentration ranging from 1 μ g/mL–100 μ g/mL (Figure 2).

Next, we examined the effect of conditioned medium (CM) on IL-8 secretion by HT-29 cells. Both CMs of BbiY and BbrY inhibited TNF- α -induced secretion of IL-8 in a dose-dependent manner (Figure 3). Concentrations of BbiY and BbrY cultured over 16 h in RPMI-1640 medium were 3.6×10^8 CFU/mL and 2.2×10^8 CFU/mL, respectively.

Nature of the inhibitory effect on IL-8 secretion in probiotic-derived CM

To investigate the nature of this soluble factor, BbiY-derived CM was subjected to molecular sieve and heat treatment. The fractions of less than and more than 3 kDa both retained the inhibitory activity toward the secretion of IL-8 in HT-29 cells, though the former fraction was greater than the latter one (Figure 4). The inhibitory effect of the latter fraction but not the former one was diminished by heat-treatment (data not shown). We checked the effect of acetic and lactic acid with their major constituents in less than 3 kDa fraction. Acetic acid but not lactic acid inhibited the IL-8 secretion in HT-29 cells, when added at the same

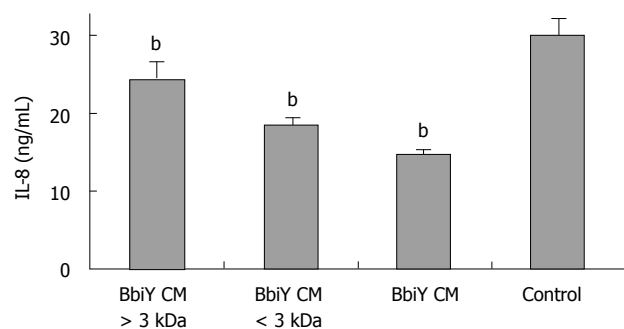


Figure 4 Assessment of the molecular weight of active component in probiotic-derived CMs. CM was separated into fractions of more than and less than 3 kDa, adjusted to the initial volume. The inhibitory effect on the secretion of IL-8 was determined as described in Figure 2. ^b $P < 0.01$ vs control.

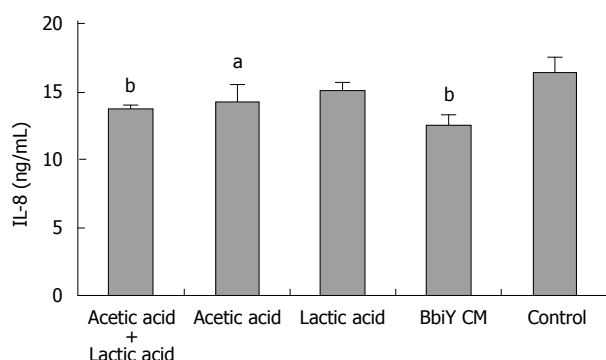


Figure 5 Effects of acetic acid and/or lactic acid on IL-8 secretion in HT-29 cells. HT-29 cells were incubated with approximately the same concentration of acetic acid (11 mmol/L) and/or lactic acid (4 mmol/L) to that of CMs. The inhibitory effect on the secretion of IL-8 was determined as described in Figure 2. ^a $P < 0.05$, ^b $P < 0.01$ vs control.

concentration as in the CM, 11 mmol/L and 4 mmol/L, respectively (Figure 5).

IL-8 and $\text{I}\kappa\text{B-}\zeta$ expression during incubation with probiotic bacteria in HT-29 cells

MRNA levels of IL-8 and $\text{I}\kappa\text{B-}\zeta$ were determined in TNF- α -stimulated HT-29 cells in the presence or absence of BbiY-derived CM (Figure 6). A repression of IL-8 expression occurred with an up-regulation of $\text{I}\kappa\text{B-}\zeta$ expression 4 h after incubation of HT-29 cells with probiotic-CM.

Probiotic DNA inhibited epithelial secretion of IL-8

HT-29 cells were stimulated with TNF- α in the presence or absence of bacterial DNA isolated from BbiY or BbrY. DNA from both BbiY and BbrY significantly inhibited the IL-8 secretion in HT-29 cells, at the concentration ranging from 1 $\mu\text{g/mL}$ -100 $\mu\text{g/mL}$, while DNA from *L. acidophilus* (YIT 0168) had no effect (Figure 7A). Treatment of probiotic DNAs with DNase abolished their inhibitory effects on the secretion of IL-8 (Figure 7B).

DISCUSSION

The efficacy of bifidobacteria-fermented milk (BFM) in the treatment of ulcerative colitis has been reported

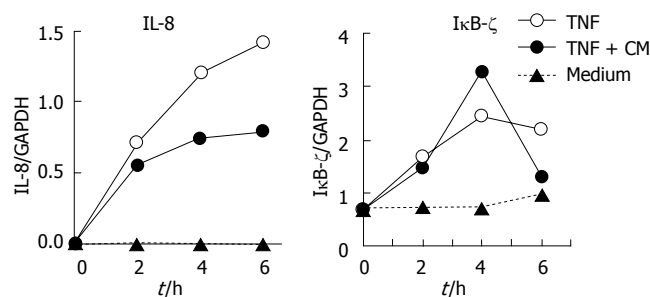


Figure 6 IL-8 and $\text{I}\kappa\text{B-}\zeta$ mRNA expression during the culture of HT-29 cells in the presence of probiotic-derived CMs. The mRNA expression was quantified as described in Materials and Methods. Results are representative of three separate experiments.

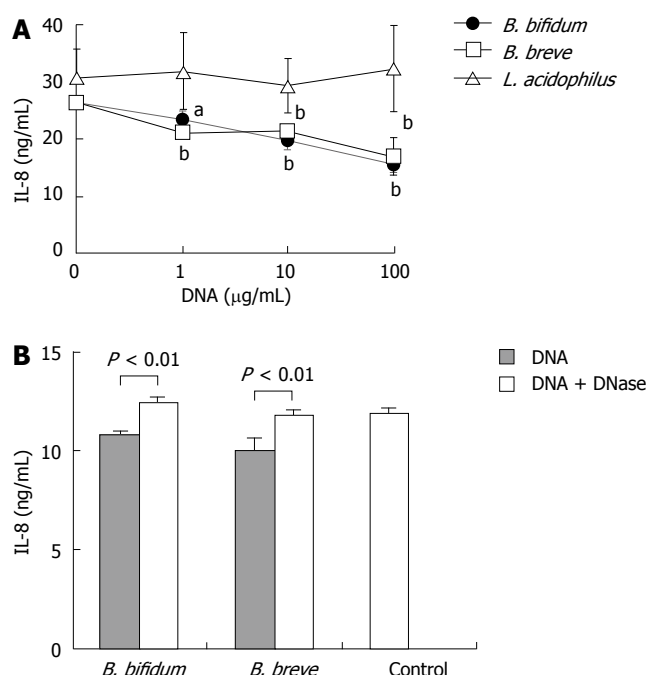


Figure 7 Effects of probiotic DNAs on IL-8 production in HT-29 cells. HT-29 cells were stimulated with TNF- α in the absence or presence of various concentrations of probiotic DNA without (A) or with DNase treatment (B). The inhibitory effect on IL-8 secretion was determined as described in Figure 2. ^a $P < 0.05$, ^b $P < 0.01$ vs control.

elsewhere^[7,8]. To determine the anti-inflammatory activity of probiotic *Bifidobacterium* strains in the BFM, we firstly investigated the effects of these strains on the production of anti-inflammatory cytokine IL-10 by PBMNC isolated from UC patients *in vitro*. IL-10 down regulates the TNF- α secretion by macrophages^[26]. IL-10 knockout mice develop IBD under conventional conditions, indicating the importance of IL-10 in the control of intestinal inflammation^[27]. It was reported that intragastric administration of IL-10-secreting *Lactococcus lactis* causes a 50% reduction in colitis in mice treated with dextran sulfate sodium^[14] and BFM containing BbiY and BbrY ameliorates IBD in SAMP1/Yit mice with an up-regulation of IL-10 synthesis and down-regulation of IFN- γ production in mesenteric lymph node cells^[28]. In this study, the two heat-killed probiotic bacterial strains in BFM induced the secretion of IL-10 in PBMNC isolated from UC patients.

It has been found that the degree of local inflammation and tissue damage in patients with IBD is dependent on the local expression of specific chemokines within affected tissues^[16]. IL-8 protein^[17] and mRNA^[18] expression are associated with inflammation in UC. The fact that conditioned media of BbY and BbrY reduced the TNF- α -induced secretion of IL-8 by HT-29 cells suggests that both probiotic strains in the BFM could produce soluble anti-inflammatory factors that inhibit IL-8 secretion in intestinal epithelial cells. No single factor produced by the probiotic strain seems to be responsible, because the fractions of less than and more than 3 kDa, differing in heat-sensitivity, were found to have inhibitory effects on IL-8 secretion in this study. Acetic acid but not lactic acid, a major component of CM, was likely to largely contribute to the inhibitory activity of the less than 3 kDa fraction. Components responsible for the inhibition of IL-8 in the more than 3 kDa fraction remain to be investigated.

I κ B- ζ is an inducible nuclear factor- κ B (NF- κ B) regulator, which is associated with the p65 and p50 subunits of NF- κ B and inhibits transcription as well as the DNA-binding of the transcription factor^[29,30]. Increased expression of I κ B- ζ mRNA in the presence of probiotic-derived CM was observed with repression of IL-8 gene expression in HT-29 cells compared to that in the absence of CM, indicating that NF- κ B-mediated IL-8 gene expression in HT-29 cells is inhibited through the induction of I κ B- ζ expression by the CM of the *Bifidobacterium* strain.

The present study has shown that the *Bifidobacterium* strains proved to be beneficial in inducing and maintaining remission of ulcerative colitis exhibit regulatory activities that contribute to the control of intestinal inflammation, thus unraveling the biological basis of the beneficial effects of probiotics in human disease.

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COMMENTS

Background

We have previously shown that treatment with bifidobacteria-fermented milk (BFM) containing live bifidobacteria is more effective than usual treatment in inducing and maintaining remission of ulcerative colitis (UC). We hypothesized that probiotic strains of Bifidobacteria in BFM may interfere with the intestinal inflammatory response as well as prevention of the overgrowth of potentially pathogenic bacteria such as *Bacteroides vulgatus*. In this study, we focused on the effect of probiotic strains on the secretion of IL-10 by peripheral blood mononuclear cells (PBMC) and also the production of IL-8 by intestinal epithelial cells.

Research frontiers

There is evidence that probiotic treatment is effective in patients with UC. The precise mechanisms by which probiotic microorganisms used in clinical trials exert their beneficial effect have not been well defined yet.

Innovations and breakthroughs

This study has shown that Bifidobacterium strains that proved to be beneficial in

inducing and maintaining remission of ulcerative colitis exhibit regulatory activities that contribute to the control of intestinal inflammation, thus unraveling the biological basis of the beneficial effects of probiotics in human disease.

Applications

Anti-inflammatory activity of probiotic bifidobacteria shown in this study supports the beneficial effects of BFM on in clinical trials. Therapeutic applications of BFM in the treatment of UC are promising.

Peer review

Probiotic Bifidobacterium strains in BFM that proved to be beneficial in inducing and maintaining remission of UC enhanced IL-10 production in PBMC and inhibited IL-8 secretion in intestinal epithelial cells, suggesting that BFM has anti-inflammatory effects against UC. The mechanism of action regarding the immunosuppressive effect of probiotics has wider therapeutic implications for the treatment of inflammatory bowel disease in general.

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Severe acute pancreatitis in the elderly: Etiology and clinical characteristics

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Abstract

AIM: To investigate the etiology and clinical characteristics of severe acute pancreatitis (SAP) in elderly patients (≥ 60 years of age).

METHODS: We reviewed retrospectively all the SAP cases treated in Xuanwu Hospital in Beijing between 2000 and 2007.

RESULTS: In 169 patients with SAP, 94 were elderly and 16 died. Biliary and idiopathic etiologies were the first two causes that accounted for over 90% of SAP in the elderly. Biliary, hyperlipemic and alcoholic etiologies were the first three causes in the young. The proportion of co-morbidity of cholelithiasis, biliary infection, hypertension and coronary heart disease in the aged was significantly higher than that in their young partners. The scores of APACHE II and Ranson were also significantly higher in the elderly except the CT score. Organ failures were more common in the elderly, but the local pancreatic complications were not different between the two groups. Mortality of the aged was correlated with the severity of SAP, multiple co-morbidity and incidence of multiple organ dysfunction syndrome (MODS). MODS was the main cause of death.

CONCLUSION: The etiology of SAP in the elderly is quite different from that in the young. Biliary and unknown factors are main causes in the aged. The elderly are subject to major organ failures but there is no difference in the occurrence of local pancreatic complications between the elderly and the young. It is crucial to monitor and improve the functions of major organs so as to prevent MODS in the aged with SAP.

INTRODUCTION

Severe acute pancreatitis (SAP) refers to the acute pancreatitis (AP) associated with organ failure and/or local complications such as necrosis, pseudocyst or abscess. The overall mortality of SAP has decreased in recent years to around 15%-20%^[1,2]. Tenner^[3] has shown in the aged, the presence of complications like infected pancreatic necrosis can increase the mortality to 50%. Fan^[4] investigated the AP in the elderly (> 75 years old) patients and indicated that the high mortality of this group is probably due to a higher prevalence of cardiopulmonary diseases and biliary stones. However, in China, the elderly refers to people aged 60 or over 60 years clinically. Few studies have compared the clinical characteristics between the elderly (≥ 60 years old) and the young patients with SAP. In this retrospective study, we aimed to investigate whether there is a difference in etiology and clinical characteristics between the aged and its younger partners with SAP, and to analyze the factors that attribute to the high mortality in the elderly.

MATERIALS AND METHODS

Patients

Between January 2000 and January 2007, 169 patients with SAP were treated in the surgery intensive care unit (SICU) and the Department of General Surgery at Xuanwu Hospital of Capital Medical University in Beijing, China. All patients' medical charts were reviewed retrospectively and the relative clinical data were collected. Acute pancreatitis was diagnosed based on the facts of the elevation of serum amylase to more than 3 times the upper normal limit and a typical clinical picture, and the

diagnostic criteria of SAP were the APACHE II score of more than 8 within 72 h after admission^[5]. Clinical severity evaluations were carried out by APACHE II and Ranson's scoring systems within 48 h after admission^[6,7]. A serial abdominal contrast-enhanced CT (CECT) was performed in each patient and the findings were graded according to the Balthazar classification^[8].

On admission, all patients were treated medically according to generally accepted principles consisting of withholding oral intake, inserting a nasogastric tube for drainage, providing pain relief, restoring fluid and electrolytes intravenously, and administration of prophylactic antibiotics. A proton pump inhibitor and somatostatin were given to prevent stress ulcers and to inhibit pancreatic excretion. The Dachengqi decoction (a Chinese herbal medicine in fluid form) was given through the nasogastric tube twice a day to improve gastrointestinal function.

Patients with gallstones were evaluated by sonography routinely. Endoscopic retrograde cholangiopancreatography (ERCP) was performed in cases of biliary etiology, as proven by ultrasonography, or magnetic resonance cholangiopancreatography and elevated bilirubin, alkaline phosphatase and aspartate aminotransferase. A papillotomy was done if stones and sludge were present in the common bile duct and some were followed by an endoscopic nasobiliary drainage. Surgical intervention (necrosectomy with drainage and continuous postoperative lavage) was performed if infected pancreatic necrosis was clinically suspected or confirmed by positive bacteriologic results of CT-guided fine-needle aspirations.

Patients consuming large amounts of alcohol were considered as having alcoholic pancreatitis. Serum triglyceride level more than 11.3 mmol/L (1000 mg/dL), and exclusion of other etiologies were accepted as the hyperlipidemic etiology^[9]. Patients were classified as having an idiopathic etiology if the history and laboratory findings ruled out any known etiologic factors, and ultrasonography revealed a normal biliary tract.

Co-morbidity was defined as a pre-existing disease or a condition in addition to the acute pancreatitis. Co-morbidity was diagnosed if the condition was an active problem and/or need routine treatment prior to the onset of acute pancreatitis. The assessment of organ function and diagnosis of multiple organ dysfunction syndrome (MODS) was performed on the basis of the Marshall multiple organ dysfunction score^[10].

Statistical analysis

Statistical evaluation was performed with SPSS 11.0 for Windows. The significant differences of clinical characteristics of the aged and young SAP patients were tested with the χ^2 test. Clinical severity scoring of SAP, such as Ranson, APACHE II and CT scorings, was described with mean \pm SD. The differences between the two groups were tested through independent samples *t* test. *P* < 0.05 was considered statistically significant.

RESULTS

General clinical characteristics

Patient general characteristics are summarized in Table 1.

Table 1 General clinical characteristics of patients with severe acute pancreatitis

	Elderly (≥ 60 yr) (<i>n</i> = 94)	Young (< 60 yr) (<i>n</i> = 75)	<i>P</i> value
Mean age (yr, range)	70.9 (60-87)	44.2 (17-59)	-
Male	44 (46.8%)	49 (65.3%)	0.0197
Female	50 (53.2%)	26 (34.7%)	0.0197
Medium ICU stay in days (range)	8 (2-56)	8 (2-90)	-
Medium hospital stay in days (range)	21 (13-64)	19 (12-90)	-
Hospital deaths	16 (17.0%)	4 (5.3%)	0.0291

Table 2 Etiology of severe acute pancreatitis *n* (%)

	Elderly (≥ 60 yr)	Young (< 60 yr)	<i>P</i> value
Biliary	61 (64.9)	28 (37.3)	0.0006
Alcohol	2 (2.1)	15 (20.0)	0.0001
Hyperlipemia	3 (3.2)	22 (29.3)	< 0.0001
Abdominal surgery	2 (2.1)	1 (1.3)	1
Drug-induced	1 (1.1)	0 (0.0)	1
Idiopathic	25 (26.6)	9 (12.0)	0.021
Total	94	75	-

There were 169 patients with SAP in this study, including 94 aged (≥ 60 years old) and 75 young (< 60 years old). The mean age of the aged was 70.9 years (range 60-87) and the young was 44.2 years (range 17-59). Of the 94 elderly patients, 44 (46.8%) were men, and 50 (53.2%) women. In patients younger than 60 years old, 49 (65.3%) were men, and 26 (34.7%) women. Medium stay in SICU was 8 d (2-56 d) in the aged and 8 d (2-90 d) in the young patients. Medium hospital stay was 21 d (13-64 d) in the aged and 19 d (12-90 d) in the young. The mortality rate of SAP was 17.0% (16 cases) in the aged and 5.3% (4 cases) in the young.

Etiology

The two major etiological factors responsible for acute pancreatitis were biliary and alcohol, although the proportions of pancreatitis attributed to these two factors varied considerably in different countries and regions. In our 169 SAP patients, biliary pancreatitis was the first common etiology in both the aged and the young groups (Table 2), but it was more common in the elderly (64.9% *vs* 37.3%, *P* = 0.0006). Interestingly, the idiopathic pancreatitis was the second most common etiology in the aged patients, significantly more common than in its younger counterparts (26.6% *vs* 12.0%, *P* = 0.0210). In patients younger than 60 years of age, the second and third causes were hyperlipemia (29.3%) and alcohol (20.0%) respectively. However, in the elderly, these two etiologies were less common (3.2% and 2.1% respectively). Two cases of pancreatitis underwent abdominal surgery in the elderly and one in the young. The operations were esophageal cancer resection, cholecystectomy and gastrectomy, respectively. One elderly woman experienced drug-induced SAP while taking Methotrexate.

Table 3 Co-morbidity of severe acute pancreatitis *n* (%)

	Elderly (≥ 60 yr)	Young (< 60 yr)	<i>P</i> value
Cholelithiasis	47 (50.0)	18 (24.0)	0.0008
Biliary infection	20 (21.3)	5 (6.7)	0.0087
Hypertension	20 (21.3)	4 (5.3)	0.0035
Coronary heart diseases	14 (14.9)	4 (5.3)	0.0490
Previous stroke	5 (5.3)	0 (0.0)	0.0667
Previous myocardial infarction	4 (4.3)	1 (1.3)	0.3836
Diabetes mellitus	8 (8.5)	9 (12.0)	0.6079
Liver cirrhosis	3 (3.2)	2 (2.7)	1.0000
Malignant diseases	4 (4.3)	1 (1.3)	0.3836
Autoimmune diseases	0 (0.0)	1 (1.3)	0.4438

Table 4 Clinical scoring of severe acute pancreatitis (mean ± SD)

	Elderly (≥ 60 yr, <i>n</i> = 94)	Young (< 60 yr, <i>n</i> = 75)	<i>P</i> value
Ranson	3.4 (1.7)	2.8 (1.6)	0.0069
APACHE II	14.0 (7.6)	9.9 (6.6)	0.0003
CT score	4.0 (1.9)	4.8 (1.8)	0.0020

Co-morbidity

Co-morbidity was defined as a pre-existing disease or condition in addition to the current onset of pancreatitis (Table 3). The morbidity of cholelithiasis, biliary infection, hypertension and coronary heart disease in the aged was significantly more common than in the young. Previous stroke was diagnosed in five aged patients but none in the young (5.3% *vs* 0%, *P* = 0.0667). Diabetes mellitus, previous myocardial infarction, liver cirrhosis, malignancies and autoimmune diseases might be seen in both groups.

Clinical scoring of SAP

In our study, three clinical scores, APACHE II, Ranson's and CT score, were collected (Table 4). The scores of APACHE II and Ranson were significantly higher in the aged than in the young (3.4 ± 1.7 *vs* 2.8 ± 1.6 , *P* = 0.0069). Surprisingly, the CT score was higher in the younger patients (4.8 ± 1.8 *vs* 4.0 ± 1.9 , *P* = 0.0020).

Organ failures and complications of SAP

Organ failures and complications were two major characteristics of SAP distinguished from mild pancreatitis. As shown in Table 5, the most frequent complications of SAP in the aged are acute lung injury (ALI) and/or acute respiratory distress syndrome (ARDS) (30.9%), followed by MODS (26.6%), electrolyte disturbances (21.3%), renal failure (18.1%), pancreatic encephalopathy (17.1%) and cardiovascular insufficiency (17.0%). Except for metabolic disorders and cardiovascular insufficiency, the incidences of ALI/ARDS, MODS, renal failure and pancreatic encephalopathy in the elderly were significantly higher than those in the young. The other complications, such as GI bleeding, paralytic ileus, pancreatic pseudocyst, pulmonary infection, fungous infection, abdominal compartment syndrome (ACS) and disseminated intravascular coagulation (DIC), occurred less frequently (< 15%) in both the elderly and the young.

Table 5 Organ failures and complications in severe acute pancreatitis *n* (%)

	Elderly (≥ 60 yr)	Young (< 60 yr)	<i>P</i> value
Total	94 (100)	75 (100)	
ALI/ARDS	29 (30.9)	13 (17.3)	0.0498
Renal insufficiency	17 (18.1)	5 (6.7)	0.0375
Cardiovascular insufficiency	16 (17.0)	11 (14.7)	0.8331
Pancreatic encephalopathy	16 (17.0)	4 (5.3)	0.0291
Hepatic insufficiency	8 (8.5)	5 (6.7)	0.7754
Metabolic disorders	20 (21.3)	12 (16.0)	0.4334
DIC	5 (5.3)	4 (5.3)	1.0000
GI bleeding	5 (5.3)	5 (6.7)	0.7521
With single organ failure	42 (44.7)	16 (21.3)	0.0019
With MODS	25 (26.6)	10 (13.3)	0.0374
Pancreatic abscess	13 (13.9)	13 (17.3)	0.6685
Pancreatic pseudocyst	11 (11.7)	8 (10.7)	1.0000
Ileus	9 (9.6)	6 (8.0)	0.7909
ACS	5 (5.3)	4 (5.3)	1.0000
Fungous infection	4 (4.3)	1 (1.3)	0.3836
Sepsis	8 (8.5)	6 (8.0)	1.0000

ALI/ARDS: Acute lung injury or acute respiratory distress syndrome; MODS: Multiple organ dysfunction syndrome; GI: Gastrointestinal; ACS: Abdominal compartment syndrome; DIC: Disseminated intravascular coagulation.

Table 6 Clinical characteristics of dead and survived cases in the aged with severe acute pancreatitis *n* (%)

		Death (<i>n</i> = 16)	Survival (<i>n</i> = 78)	<i>P</i> value
Gender	Male	9 (56.3)	35 (44.9)	0.4255
	Female	7 (43.7)	43 (55.1)	0.4255
Etiology	Biliary	10 (62.5)	51 (65.4)	1.0000
	Idiopathic	5 (31.3)	20 (25.6)	0.7570
Scoring (mean ± SD)	Ranson	5.6 ± 1.8	3.1 ± 1.3	< 0.0001
	APACHE II	26.3 ± 9.3	11.9 ± 4.7	< 0.0001
	CT score	5.6 ± 2.1	3.7 ± 1.7	0.0001
Co-morbidity	Single	4 (25.0)	28 (35.9)	0.5647
	Multiple	12 (75.0)	24 (30.8)	0.0015
Organ dysfunction	Single	1 (6.3)	16 (20.5)	0.2882
	MODS	15 (93.8)	10 (12.8)	< 0.0001

Mortality of SAP in the elderly

The mortality of SAP in the aged was significantly higher than that of the young in our data (17.0% *vs* 5.3%, *P* = 0.0291, Table 1). We further compared the clinical characteristics of the dead and the surviving patients of the elderly (Table 6). We found no difference in either gender or etiologies between the two subgroups. Only the severe scores, multiple co-morbidities and MODS were significantly higher in the subgroup of the dead patients and almost all of this subgroup died of MODS.

DISCUSSION

In most studies, the two major etiological factors, biliary disease and alcohol abuse together account for more than 80% of AP patients^[11,12]. Studies in the United Kingdom reveal that 40%-57% of patients with AP have small gallstones^[13,14].

In our study, biliary pancreatitis was the most common

cause for SAP in both aged and young patients. However, biliary etiology in the elderly is as high as 64.9%, we thought it was because very few patients were addicted to alcohol and the incidence of cholelithiasis increased in this subgroup. Meanwhile, unknown etiology, or idiopathic pancreatitis accounted for 26.6% of the elderly and ranked second, quite different from the etiologies in the young patients. This is similar with other reports in which 30%-40% of elderly patients with acute pancreatitis have an unclear etiology^[15]. Interestingly, the second more common etiology was not alcohol abuse but hyperlipemia in the young patients in our study. Although alcoholism was associated with over 80% of patients in studies from New York and around 70% in Scandinavian countries, in the Beijing area, it seems not a major concern.

Many drugs are associated with the development of acute pancreatitis. These include didanosine, furosemide, corticosteroids, azathioprine and sodium valproate^[16]. However, there is no clinical feature that can differentiate drug-induced pancreatitis from other factor caused-pancreatitis. Drug-induced pancreatitis is usually diagnosed using the following criteria: pancreatitis developed during treatment, resolved following the discontinuation of the drug, and re-developed following re-challenge of the offending drug^[17]. Because no causal factor for pancreatitis was found after the initial work-up, acute pancreatitis induced by Methotrexate was suspected in a 63 years old woman who took it for a week and her symptom was relieved after cessation of the drug.

Even though the mortality of SAP is around 22%-30%, few data showed the mortality of SAP in the elderly. A Japanese survey done from 1991 to 1995 showed that the mortality of SAP is greater than 20% in patients over 50 years of age^[18]. Andersson reported that the hospital mortality of AP in a university hospital in Sweden decreased slightly, from 4.7% (1975-1985) to 3.7% (1986-1996), and that the average age of the dead patients markedly increased from 59.2 to 73.6 years^[19]. This suggested that the older the patients, the higher mortality they have. Fan^[4] has concluded that mortality from AP was related to coexisting diseases in the elderly, not to the severity of acute pancreatitis and reported a 21.3% mortality from AP in patients aged above 75 years. Browder^[15] also believed organ function compromise correlates with mortality and appears more significant than severity of pancreatic disease. In our study, the co-morbidities, including cholelithiasis, biliary infection, hypertension and coronary heart disease were more common in the aged. The mortality of SAP in this group was 17%, but 5.3% in the young.

Recently, Kaya^[20] demonstrated that the APACHE II score is the best predictor of mortality among Ranson, Imrie and APACHE II scores. We found that both APACHE II and Ranson's criteria were of significance in predicting mortality in the aged. Unexpectedly, the CT score was different from the two above scores, and it was lower than in the aged patients. This seems that the average local pathological conditions in the aged were less serious than that in the young patients. To explain this data with caution and to exclude the heterogeneity of the aged group, we further analyzed the dead and the living cases in the elderly. The CT score was markedly higher

in the deceased cases, suggesting that it is still a sensitive predictor for mortality from SAP. Leung^[21] reported that the sensitivity of CT severity index (CTSI) is higher than Ranson score and APACHE II score, although they are also the predictors for complications, mortality and the length of AP course. Some researches suggest that MRI seems to be a reliable method of staging AP severity in comparison with CECT scan^[22]. Otherwise, C-reactive protein (CRP) is a useful indicator for assessing severity and recommended to apply in clinical practice^[23,24].

The most frequent complication of SAP in the aged in our study is ALI/ARDS (29 cases, 30.9%), followed by MODS (25, 26.6%), electrolyte disturbances (21.3%), renal failure (17, 18.1%), pancreatic encephalopathy (16, 17.0%) and shock (16, 17.0%). Currently, there are no specific examinations or unified diagnostic standards for pancreatic encephalopathy. It results from the combined actions of multiple factors based on cerebral demyelination due to pancreatin^[25]. In our study, it was diagnosed according to clinical symptoms and electroencephalogram or MRI.

The local pancreatic complications, such as pancreatic abscess, pancreatic pseudocyst and ACS, are not different between the two groups. It supports the opinion that the local pathological condition is not the critical factor but organ dysfunction, especially cardiovascular, pulmonary, renal and cerebellar function, for the high incidence of death in the elderly. This phenomenon could be related to the progressive decline in physiological function of major organs with aging. The other complications, such as alimentary tract hemorrhage, pulmonary infection, fungous infection and disseminated intravascular coagulation (DIC), do not occur so frequently (< 15%) and there is no difference between the elderly and the young. We suggest that in treatment of SAP in the aged, priority should be given to monitoring and improving the functions of major organs so as to prevent MODS. At the same time, it is necessary to find out the origin of SAP and remove it early by minor-injury method. Additionally, the fluid quantity and speed of transfusion should be decreased in the aged and the dose of medicine should be used properly in order to avoid injury of the organ function.

In our study, the incidence of paralytic ileus was less than 10%. It may be associated with the gastrointestinal functions. SAP patients admitted to our intensive care unit were given purgatives, such as magnesium sulfate, Mannitol, lactulose and Dachengqi decoction (Chinese herbal medicine). These drugs could promote gastrointestinal motility, ameliorate intestinal permeability and decrease bacterial translocation^[26,27]. They can also improve bile discharge and decrease effusion in the abdominal cavity. Rhubarb is the main constituent of Dachengqi decoction. Rhubarb can exert protective effects on SAP by inhibiting the inflammation of pancreas in rats, improving pancreatic microcirculation and altering exocrine secretion^[28]. Wu^[29,30] also reported the benefit from Chinese herbal medicine in treatment of SAP and recommended the regimen for clinical use.

In conclusion, SAP in the aged had obviously different features from the young. Both of biliary and unknown etiological factors result in over 90% of SAP in elderly. The co-morbidities of cholelithiasis, biliary

infection, hypertension and coronary heart disease are more common than in the young. APACHE II scores and Ranson's criteria are markedly higher in the elderly and CT scores much higher in the elderly who died in the hospital. Therefore, the mortality of the aged patients is correlated with the severity of SAP, and co-morbidity and incidence of MODS are not relevant to patients' gender or etiologies. Importance should be attached to monitoring and improving the functions of major organs so as to prevent MODS in the aged with SAP.

COMMENTS

Background

The overall mortality of severe acute pancreatitis (SAP) has decreased in recent years to around 15%-20%. Few studies compared the clinical characteristics between the elderly (≥ 60 years old) and the young patients with SAP.

Research frontiers

Some researches have shown that the presence of complications such as infected pancreatic necrosis, in the aged (> 75 years old) can increase the mortality of AP to 50%, probably due to a high prevalence of cardiopulmonary diseases and biliary stones.

Innovations and breakthroughs

The authors retrospectively reviewed the SAP cases that were treated in a hospital in Beijing between 2000 and 2007 to investigate the etiology, clinical characteristics and outcomes of SAP in the elderly patients.

Applications

The authors suggest that importance should be attached to monitoring and improving the functions of major organs so as to prevent MODS in the aged with SAP.

Peer review

This article reviews the experience with elderly patients in China with SAP. This is an interesting and well-written paper.

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RAPID COMMUNICATION

Prognostic factors for progression of liver structural lesions in chronic hepatitis C patients

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Abstract

AIM: To evaluate the epidemiological, clinical, laboratory and histological variables capable of predicting the progression of hepatic structural disturbances in chronic hepatitis C patients during the time interval between two liver biopsies.

METHODS: Clinical charts of 112 chronic hepatitis C patients were retrospectively analyzed, whereas liver biopsies were revised. Immunohistochemical detection of interferon receptor was based on the Envision-Peroxidase System.

RESULTS: In the multivariate analysis, the variables in the age at first biopsy, ALT levels, presence of lymphoid aggregates and siderosis were the determinants of the best model for predicting the severity of the disease. The direct progression rate of hepatic structural lesions was significantly higher in untreated patients, intermediate in treated non-responders and lower in treated responders to antiviral therapy (non-treated *vs* responders, 0.22 ± 0.50 *vs* -0.15 ± 0.46 , $P = 0.0053$). Immuno-expression of interferon receptor is not a relevant factor.

CONCLUSION: The best predictors of the progression of fibrosis are age at the first liver biopsy, extent of ALT elevation, inflammation at liver histology and hepatic siderosis. Antiviral treatment is effective in preventing the progression of liver structural lesions in chronic hepatitis C patients.

INTRODUCTION

Studies on hepatitis C virus (HCV) in Brazil indicate a moderate prevalence in the country as a whole. The prevalence of HCV infection in the city of São Paulo^[1] was estimated at 1.4%, ranging from 0.7% to 2.12% according to age, geographical region and socioeconomic characteristics. The most prevalent genotype in North America, comprising approximately 60% of all cases of HCV, is genotype 1, followed by genotypes 2 and 3. In Brazil, genotype 1, subtype 1b is also the most prevalent except in the south of the country where there are more cases of infection by genotype 3^[2-4].

Hepatic cirrhosis and hepatocellular carcinoma (HCC) are consequences of chronic hepatitis C. It is estimated that HCC will develop in 1%-4% of patients per year in the first five years following the onset of hepatic cirrhosis^[5]. The mean interval from the time of infection to onset of cirrhosis is approximately 30 years, but cirrhosis may occur within a range of 10-50 years^[6]. In the majority of the patients, progression of the disease involves fibrosis, its extension in the hepatic tissue being the determinant of more severe clinical events in patients with HCV^[6]. Once cirrhosis is established, it represents an irreversible condition, and strategies to avoid the progression of fibrosis are essential in order to avoid progressive liver dysfunction^[7]. During the chronic infection, 30% of the patients will evolve asymptotically with no significant fibrosis or evidence of serious hepatic dysfunction even in the presence of persistently high enzyme levels^[8]. In another 30% of cases, the persistently elevated aminotransferase levels will result in fibrogenesis and progressive liver dysfunction. In

10% cases, the clinical course is variable.

Previous studies have identified some factors associated with the worst evolution of chronic hepatitis C, such as age > 40 years at the time of infection and alcohol intake > 50 g/d, as independent factors for the worst prognosis^[9]. Controversial reports on elevated alanine aminotransferase (ALT) levels as a predictive factor for increased histological deterioration have been published in the literature; however, increased histological deterioration has been described as related to apoptosis in patients with chronic hepatitis C and normal ALT^[10].

Hepatic fibrogenesis is a result of the action of several aggressive factors on the liver. Various cells are involved in this process, including Kupffer cells (liver macrophages), sinusoidal endothelial cells, hepatocytes and stellate cells (Ito cells or lipocytes). Hepatic stellate cells play a key role in the development of fibrosis and are the major source of extracellular matrix. There is approximately one such cell for every six hepatocytes^[7]. Chronic hepatitis C is defined as the continuous hepatic aggression associated with the elevation of aminotransferases or positive viral markers for periods longer than 6 months. Various consensus has concluded that treatment modality should usually be based on structural alterations and hepatic necroinflammatory activity status^[11]. The purpose of a biopsy in the pretreatment phase is different in those patients with moderate inflammation and an advanced level of fibrosis for whom treatment would be indicated, and in those with mild inflammation and an absence or minimal portal fibrosis in whom treatment would be of little use. The French Cooperative Group METAVIR^[12] proposed a scoring system to distinguish fibrosis of the portal spaces from that associated with the lobular-central vein, and graded the stage of portal fibrosis on a five-point scale, consisting of 2 different grades in the septum and 3 grades in the central lobular veins according to the amount of fibrosis and its association with perisinusoidal fibrosis. According to the METAVIR scoring system, patients with fibrosis stages F2, F3 and F4 should be considered for antiviral therapy. The objective of studying serial biopsies is to evaluate the progression of liver damage in chronic HCV. Several parameters have been used in the staging of hepatic disease at the tissue level. The ideal staging system should evaluate each histological component separately such as piecemeal necrosis, confluent necrosis, lobular activity and portal inflammation. Among the patients treated with IFN in various regimens and with various drug combinations, a significant number failed to respond to treatment, while of those who did respond to treatment, some experienced a relapse. Several virological variables such as HCV genotypes 2 and 3 and low HCV RNA load, as well as other variables related to the host such as young age, short disease duration and absence of cirrhosis, have already been cited as being directly related to a higher chance of responding to IFN. Data in the literature on the ability of IFN receptors to predict response rates are sparse. Yatsuhashi *et al.*^[13] studied 55 patients with chronic HCV infection treated with IFN- α for 16 wk at a dose of 6 million units/day in the first two weeks followed by 6 million units three times per week up to the end of treatment. The patients not responding to the treatment

were those with lower hepatic IFN receptor expression.

The aim of this study was to evaluate the epidemiological, clinical, laboratory and histological variables that may be predictive of the progression of fibrosis in chronic hepatitis C patients during the interval between two liver biopsies.

MATERIALS AND METHODS

Patients

Epidemiological, clinical, laboratory and histological data were retrospectively analyzed from 112 patients with chronic hepatitis C, receiving care at the Hepatology Clinic of the Department of Gastroenterology, *Hospital das Clinicas*, University of São Paulo School of Medicine (São Paulo, Brazil). The study was approved by the institute's Internal Review Board and was carried out in accordance with the Helsinki Declaration. The patients had been subjected to two liver biopsies at two different periods of time. The first biopsy was performed between March 1992 and January 2002. The patients received or did not receive treatment with interferon alpha-2 and ribavirin for 6-12 mo according to the genotype. Patients with non-genotype 1 HCV were treated for 6 mo following the first biopsy, while those with genotype 1 or when genotype was unknown at the time of the initial biopsy received treatment for a year. The patients who remained HCV RNA negative for 6 mo or longer after the treatment were considered responders.

Inclusion criteria: Chronic hepatitis C, defined by the presence for at least 6 mo of serum anti-HCV antibodies, as confirmed by ELISA (Enzyme-Linked Immunosorbent Assay) and HCV RNA, evaluated using qualitative and quantitative methods in a reverse transcription polymerase chain reaction assay; adults over 18 years of age whose medical charts were available at the time of the first liver biopsy; no HCV treatment prior to the first biopsy.

Exclusion criteria: History of prior HCV treatment, hepatitis B virus (HBV) infection; HIV infection or AIDS; chronic renal failure; patients with transplants; cancer patients or those in use of any immunosuppressive drugs; alcohol use (> 20 g/d); autoimmune and metabolic diseases of the liver; schistosomiasis mansoni; abnormalities in serum alpha-fetoprotein levels suggesting primary hepatic neoplasia; and use of any hepatotoxic drug.

Histopathological evaluation

Fragments of liver tissue were obtained by percutaneous needle biopsy before and after therapy in the treated group and on two different occasions according to clinical indication during follow-up in the untreated group. In all biopsies, sections were stained with hematoxylin and eosin (H&E), Masson's trichrome, Perl's Prussian blue and reticulin stain, and re-evaluated blindly by a single pathologist. Image analysis was done with Image Pro-Plus 4.5.1 software (Media Cybernetics, Bethesda, MD, USA). By evaluating the natural history of chronic hepatitis C infection in the interval between the two biopsies, it was possible to predict whether evolution would be mild or

severe. Severe histopathological disease was defined as necroinflammatory activity ≥ 2 ($A \geq 2$) and/or fibrosis ≥ 2 ($F \geq 2$) in accordance with the classification defined by POYNARD *et al*^[14].

To predict the evolution of hepatic structural disturbances in the interval between the two biopsies, the presence or none histological findings of ductal lesion, lymphoid aggregates, steatosis and siderosis were assessed in the first biopsy.

Necroinflammatory activity was graded by integrating the intensity of piecemeal and lobular necrosis as proposed by the METAVIR group^[15]. A0: No histologic necroinflammatory activity; A1: Minimal histologic necroinflammatory activity; A2: Moderate histologic necroinflammatory activity; and A3: Severe histologic necroinflammatory activity.

The levels of structural changes were defined following the scoring system by the METAVIR group^[15]. F0: No fibrosis; F1: Portal fibrosis without septa; F2: Portal fibrosis with few septa; F3: Numerous septa without cirrhosis; and F4: Cirrhosis.

The progression of hepatic architectural disturbances was assessed according to the METAVIR scoring system^[14]. In particular, the estimated (indirect) progression of fibrosis was calculated from the ratio between the stage of fibrosis at the first biopsy and the time after infection, i.e. the estimated duration of the infection in years, expressed in METAVIR units of fibrosis per year (METAVIR FU/year). In this model, it is assumed that at the time of infection the patient had no hepatic fibrosis (F0) and from that day on, the progression of fibrosis was constant. In those patients in whom the time of infection was unknown, it was impossible to calculate the indirect progression of fibrosis. In order to evaluate these cases correctly, we excluded patients with any other possible epidemiological causes of fibrosis (see exclusion criteria). The direct progression of fibrosis over time was defined as the difference in the stages of fibrosis between the first and the second biopsy, i.e. progression during the interval of years between the two biopsies, with the final result expressed in fibrosis units per year.

IFN receptor immunohistochemical detection

Whenever there was sufficient remaining tissue available from the paraffin block, two histological slides for each patient were submitted to immunohistochemical detection of interferon receptor, using a monoclonal antibody (clone ANOCK 4866; Otsuka Pharmaceutical, Tokushima, Japan) which was kindly provided by Dr. Michitami Yano of the Institute for Clinical Research, Nagasaki Chuoh National Hospital, Japan. As previously standardized, antibody dilution was 1:100 followed by the dextran polymer-peroxidase Envision System (Dako, Carpinteria, CA). The results were expressed as positive or negative, and a reaction was considered positive when hepatocytes showed cytoplasmic reactivity.

Serum biochemical analysis

Laboratory biochemical tests were performed by standard methods using automated techniques (Modular P800;

Roche Diagnostics; Indianapolis, IN, USA).

Statistical analysis

Descriptive analysis was carried out for both continuous and qualitative variables, obtaining means or medians and frequencies or percentages, respectively. The results were then correlated with the treatment. For analysis of the prognostic factors related to the progression of the mild or severe forms of the disease throughout its natural history at the time of the first biopsy, univariate and multivariate methods were applied. In the univariate analysis, Student's *t* test for independent samples was used in the evaluation of continuous variables, and the Kruskal-Wallis test was applied when the variables were nominal or continuous with non-normal distribution. Pearson's χ^2 test or Fisher's exact test were also used in the evaluation of proportions between the categorical variables. Multivariate techniques were applied to evaluate whether significant variables in the univariate analysis were able to predict the mild or severe forms of hepatic disease. Logistics regression with *forward selection* using the likelihood ratio test was used to identify significant variables. Significance was established at $P \leq 0.05$.

RESULTS

The general features of the patients in this study are described in Table 1. Table 2 shows the results of the univariate analysis in which age at first biopsy, duration of the infection, ALT levels, albumin, prothrombin activity, lymphoid aggregates and siderosis were identified as potential candidates for the multivariate analysis.

The variables were studied in various models and the final model was obtained through progressive comparison using the likelihood ratio test to identify the most adequate and stable model capable of distinguishing between progression to mild or severe liver disease. The variables including age at first biopsy, ALT levels, lymphoid aggregates and siderosis, were determinants of the best model for predicting the severity of the disease (Table 3).

The indirect progression to hepatic fibrosis was evaluated in all patients. Patients with more severe forms of the disease had a significantly higher mean progression rate than those with mild disease ($P < 0.0001$) (Table 4).

Regarding the direct progression of hepatic fibrosis, a total of 112 patients underwent two liver biopsies. Following the first biopsy, 79 of these patients were treated, while 33 received no treatment. The direct progression of hepatic fibrosis was different (0.2184 ± 0.4987) in the groups of untreated patients, treated non-responders and treated responders ($P = 0.01$) as seen in Table 5. Untreated patients had higher progression rates contrasting to lowest rates in those who responded to antiviral treatment (-0.1459 ± 0.4584). It is important to acknowledge that even the non-responders to the anti-viral treatment were benefited, showing herein intermediate rates of progression of liver structural disturbances (0.0382 ± 0.3661). There was a clear reduction in the progression of fibrosis in those treated patients. Compared with the untreated patients, treated non-responders and treated responder groups, the

Table 1 Clinical and laboratory features of patients at first biopsy

Variables	Values
Number of patients	112
Age in years (mean \pm SD)	46.9 \pm 12.3
Gender (Male/Female)	65/47 (58%/42%)
Age at infection in years (mean \pm SD)	25.8 \pm 13.1
Route of infection	
Blood transfusion	52/112 (46.4%)
Illegal drug use	13/112 (11.6%)
Other known routes	28/112 (25.0%)
Unknown	19/112 (17.0%)
Duration of infection in years (mean \pm SD)	22.7 \pm 10.8
Laboratory	
Alanine aminotransferase (\times UNL)	2.96 \pm 2.43
Aspartate aminotransferase (\times UNL)	1.74 \pm 1.16
Gamma-glutamyltranspeptidase (\times UNL)	1.28 \pm 0.97
Serum albumin (mg/dL)	4.32 \pm 0.51
Prothrombin activity (%)	90.2 \pm 9.9
Platelet count (/mm ³)	216 510 \pm 68 088
Genotyping of HCV	
1	73/105 (69.5%)
Non-1	31/105 (29.5%)
Indeterminate	1/105 (0.9%)
Liver Histopathology	
Lymphoid aggregates	54/112 (48.2%)
Ductal injury	13/112 (11.6%)
Steatosis	58/112 (51.8%)
Siderosis	20/112 (18.0%)
IFN Receptors	79/110 (71.8%)
Fibrosis (METAVIR scoring system)	
F0	19/112 (17.0%)
F1	41/112 (36.6%)
F2	25/112 (22.3%)
F3	6/112 (5.4%)
F4	21/112 (18.8%)
Histological Activity (METAVIR scoring system)	
A0	27/112 (24.1%)
A1	53/112 (47.3%)
A2	24/112 (21.4%)
A3	8/112 (7.1%)
Treatment	
Non-treated	33/112 (29.5%)
Treatment responders	23/112 (20.5%)
Treatment non-responders	56/112 (50.0%)

UNL: Upper normal limit.

relationship between direct progression of hepatic fibrosis (according to the METAVIR scoring system) and IFN receptor-positivity (by immunohistochemistry) was not predictive of response to treatment. Although necroinflammatory activity at the first biopsy was not a determinant of the direct progression of fibrosis, a good correlation was observed between progression of necroinflammatory activity (defined as the difference in activity between the first and the second biopsy) and direct progression of fibrosis ($P = 0.03$).

DISCUSSION

According to the model described by Poynard *et al.*^[9], the annual rate of fibrosis progression can only be calculated in those patients with a clear duration of infection prior to the first liver biopsy (natural history). Therefore, the uni- and multivariate analysis could only be performed in

Table 2 Univariate analysis of factors associated with presence of significant hepatic disease according to liver biopsy in patients with chronic HCV infection

Variables	Odds ratio	95% CI	P
Age at first biopsy (yr)	1.0853	1.0370-1.1357	0.001
Duration of infection	1.0593	1.0198-1.1004	0.003
Gender	1.2719	0.5553-2.9132	0.569
Illegal drug use	0.6639	0.2241-1.9666	0.460
Blood transfusion	0.5792	0.2525-1.3284	0.197
ALT	1.2000	0.9808-1.4682	0.076
AST	1.4133	0.9681-2.0633	0.073
GGT	1.2978	0.8178-2.0594	0.268
Albumin	0.3660	0.1556-0.8604	0.021
Prothrombin activity	0.9252	0.8797-0.9730	0.003
Platelets	0.9999	0.9999-1.0000	0.107
Steatosis	1.8571	0.8110-4.2525	0.143
Lymphoid aggregates	3.9747	1.6612-9.5081	0.002
Ductal injury	1.9523	0.4574-8.3329	0.366
Siderosis	9.5454	2.0376-44.7150	0.004
IFN receptors	0.4285	0.1679-1.0938	0.076
Infection duration	0.9975	0.9606-1.0358	0.898

Table 3 Logistic regression analysis to identify most appropriate models for predicting progression of disease

Variables	Activity or fibrosis ($A \geq 2$ and/or $F \geq 2$)		95% CI	
	Odds ratio	P	Lower	Upper
Age at first biopsy (yr)	1.1045	0.001	1.0463	1.1659
ALT	1.3333	0.021	1.0450	1.7011
Lymphoid aggregates	4.8340	0.005	1.6118	14.4979
Siderosis	6.4875	0.032	1.1748	35.8233

Table 4 METAVIR indirect progression of fibrosis in patients with chronic hepatitis C according to histologic severity

	Liver fibrosis progression (fibrosis units/yr)	
	n	mean \pm SD
Mild disease	45	0.0356 \pm 0.0552
Severe disease	48	0.1733 \pm 0.1424
Total	93	0.1067 \pm 0.1289

Student's *t*, $P < 0.0001$.**Table 5** METAVIR direct progression of structural disturbances in patients with chronic hepatitis C

Treatment	n	Direct fibrosis progression (fibrosis units/yr)			
		Mean	SD	Median	25%-75%
Non-treated	33/112 (29.4%)	0.2184	0.4982	0	0.000-0.500
Non-responders	56/79 (70.9%)	0.0382	0.3661	0	0.000-0.000
Responders	23/79 (29.1%)	-0.1459	0.4584	0	-0.461-0.000

$P = 0.0117$; Kruskal-Wallis; Non-treated *vs* treated, $P = 0.0270$; Non-responders *vs* Responders, $P = 0.0344$; Non-treated *vs* responders, $P = 0.0053$; Non-treated *vs* non-responders, $P = 0.1413$. Mann-Whitney/Wilcoxon.

93 of the 112 patients. Since none of these patients had been treated previously, excluding other causes of fibrosis, fibrosis was assumed to have been absent at the time of initial infection. Poynard *et al.*^[9] analyzed 2235 patients with

chronic hepatitis C and found a mean fibrosis progression rate of 0.133 FU/year, similar to the mean rate found in this study (0.106 FU/year). On the other hand, in 2003, Ghany *et al*^[16] reported a fibrosis progression rate of 0.44 FU/year. Considering the difficulty in identifying other fibrosis markers, various clinical, epidemiological, laboratory and histological variables have been evaluated to define their relevance in the natural history of chronic hepatitis C^[17]. Using uni- and multivariate analysis, Poynard *et al*^[18] reported that age at first liver biopsy was higher in those patients who progressed to severe disease ($P < 0.001$; OR:1.09), mean age at first biopsy being 53.4 ± 10.6 years for patients with severe disease and 43.8 ± 10.9 years for patients with mild disease. Previous studies have described the duration of infection and the occurrence of fibrosis as relevant factors associated with progressive liver dysfunction. Verbaan *et al*^[19] described fibrosis (rather than histological activity) as a factor related to the duration of infection; however, in the present study, no difference was found in the duration of infection between the groups with severe and mild disease.

On the other hand, the mean age of the patient at infection was significantly different between the two groups ($P < 0.004$). The univariate analysis showed the mean age at infection of 30.2 ± 13.4 years in the group that progressed to severe disease, was almost 10 years higher than those with mild disease. Poynard *et al*^[9] observed a worse evolution of hepatitis C in patients infected after the age of 40 years. It is possible that in the present study, the age at first biopsy was statistically significant as an independent predictor of a more severe disease because of the older age at infection rather than the duration of the infection. In addition, most of these patients were under 40 years of age. In our series, age at first biopsy was a significant variable in the logistic model. Tassopoulos *et al*^[20] also found that patients with mild chronic hepatitis were younger than those with moderate to severe diseases, with mean ages of 41 and 45 years, respectively.

Although there was a tendency towards significance, elevated levels of alanino aminotransferase (ALT) and aspartate aminotransferase (AST) were not found to be predictive of the evolution to mild or severe disease. Mathurin *et al*^[21] evaluated 204 untreated patients prospectively, and found slower fibrosis progression in those with normal ALT. Koda *et al*^[22] developed the FibroIndex, which is derived from the platelet count, AST and gamma globulin measurements. The authors concluded that this is a simple and reliable index for predicting significant fibrosis in chronic hepatitis C. In our study, elevated GGT failed to identify patients with severe disease at the first biopsy. Mathurin *et al*^[21] described significantly elevated GGT in patients with abnormal ALT levels, which was in turn correlated with higher fibrosis scores.

Platelet count was not predictive of the severity of liver disease. Our results contradict the data reported by Poynard *et al*^[18] showing the age of the patient and platelet count as being independent factors for progression to severe liver disease. Moreover, Ghany *et al*^[16] described platelet count as a factor for differentiating early from

advanced stages of liver disease. The lack of statistical significance for platelet count as a predictor in the present study may be due to the inclusion of cases of fibrosis grade 2 (F2) in the severe group in contrast to the findings of previous studies reporting a correlation between platelet count and advanced liver disease with fibrosis 3 (F3) and 4 (F4). Ohta *et al*^[23] developed a simple surrogate index consisting of platelet count and albumin level, which reflect the histological fibrosis stage of patients with chronic hepatitis C.

Steatosis was not related to severe disease in 93 patients of this study. Some authors reported a higher frequency of steatosis in patients with genotype 3 HCV^[24,25]. Westin *et al*^[26] studied 98 treatment-naïve patients and described a higher fibrosis progression rate in those with steatosis and genotype 3 HCV. Wyatt *et al*^[27] reported that steatosis is strongly associated with fibrosis and tends to increase over time, but is reduced in patients developing cirrhosis.

Other histological variables such as interferon receptors failed to correlate with the evolution of the disease. Interferon receptor expression is associated with a higher response to interferon treatment^[13]; however, no analysis has been carried out taking the natural history of the disease into consideration. Lower receptor expression would be expected in the group with worse evolution. The lack of significance may be due to the qualitative method used, and further studies are necessary since patients with liver cirrhosis are known to be less responsive to interferon treatment^[28].

The presence of siderosis at the first biopsy was correlated with the evolution of liver disease, with an odds ratio of 9.54 in the univariate and 6.48 in the multivariate analysis. This variable has been reported by some authors to be associated with higher fibrosis progression^[29].

The presence of lymphoid aggregates had an odds ratio of 3.97 for evolution of disease in the univariate analysis. After the logistic regression model, this factor persisted as a significant factor, with an odds ratio of 4.83. Similar results have also been observed by Delladetsima *et al*^[30].

Univariate analysis showed that the time interval between the two liver biopsies was similar in the three groups: untreated patients, non-responders and responders to treatment. The direct fibrosis progression rate was higher in the untreated group followed by the non-responder and responder groups. However, when the mean fibrosis progression rates of the untreated and non-responder groups were compared, no significant difference was found between the two groups. The direct fibrosis progression rates were 0.0382 and 0.2184 FU/year, respectively ($P < 0.15$). Sobesky *et al*^[31] previously found a higher direct fibrosis progression rate in untreated patients and a lower progression rate in patients who responded to treatment, while the progression rate was slightly lower in patients who did not respond to treatment. Similarly, Poynard *et al*^[15,32] reported a lower progression rate of fibrosis in treated patients, which was correlated with virological response and duration of treatment. In addition, an improvement at histology was described in patients who responded to treatment.

Interferon receptor expression is associated with a greater response to interferon treatment^[13]. The presence

of receptors would increase the probability of response to both endogenous and synthetic interferon, resulting in reduced progression of fibrosis. However, this was not observed in this study. The lack of correlation between interferon receptor expression and response to treatment may be explained by the qualitative method used in this study in contrast to the quantitative methods used in other studies^[13].

Elevated levels of ALT and progression of liver fibrosis has been previously described^[33,34]. However, in this study, elevated ALT levels were not predictive of a higher direct progression rate of fibrosis, probably due to the effect of treatment since the patients with higher ALT values were in the treated group.

Histological variables failed to correlate with the direct progression of fibrosis. Even siderosis, an independent factor for indirect fibrosis progression in the multivariate analysis, failed to show any association with direct fibrosis progression.

Although steatosis was not associated with fibrosis progression in this study, Westin *et al.*^[26] gave a higher direct fibrosis progression rate in patients with genotype 3 HCV and steatosis.

The patients with deteriorated necroinflammatory activity at histology had a mean direct fibrosis progression rate of 0.1883 FU/year, while patients with histological improvement had a rate of 0.0751 FU/year. These results indicate a good correlation between direct fibrosis progression rate and histological activity because fibrosis might be a result of the necroinflammatory activity.

In conclusion, among untreated chronic hepatitis C patients (natural history), the mean fibrosis progression rate was 0.036 ± 0.06 METAVIR units per year in those with mild disease and 0.17 ± 0.14 in those with severe disease. The best predictors of fibrosis progression are: age at first liver biopsy, the extent of ALT elevations, inflammation at liver histology and hepatic siderosis. The other factors, such as interferon receptor expression, are not significantly associated. When the histological progression rate is evaluated between 2 liver biopsies, the progression of fibrosis in METAVIR units per year is higher in non-treated patients and lower in those patients who responded to treatment. The worst histological evolution is correlated with the highest progression rate of fibrosis. No laboratory or histological variable is able to predict the evolution of fibrosis between two liver biopsies.

COMMENTS

Background

Chronic hepatitis C virus (HCV) infection may progress to cirrhosis over an average of 30 years. Once cirrhosis is established, it represents an irreversible condition, and strategies to avoid the progression of fibrosis are essential in order to avoid progressive liver dysfunction. The extent of inflammation, neovascular formation and fibrosis are debated as possible determinants of more severe clinical course of HCV.

Research frontiers

Hepatitis C virus infection is often referred to as "the Silent Epidemic", because the progression to symptomatic liver disease may take decades. The clinical profiles in chronic hepatitis C are highly heterogeneous in terms of severity and progression rates towards end stage complications. Therefore staging and prognostic

assessment in the individual case would be a valuable strategy to identify patients at higher risk of progression.

Innovations and breakthroughs

This study identified age at first liver biopsy, extent of ALT elevation, inflammation at liver histology and hepatic siderosis as the best predictors of the progression of fibrosis. Antiviral treatment was effective in preventing progression of liver structural lesions in patients with chronic hepatitis C.

Applications

The mean interval from time of hepatitis C virus infection to onset of cirrhosis is approximately 30 years, but cirrhosis may occur within a range of 10-50 years. The results of our study contribute to a better understanding of the predictors of progression of fibrosis in chronic hepatitis C and will help identify patients at higher risk of rapid progression of the disease.

Terminology

The progression of hepatic structural disturbances was assessed according to the METAVIR scoring system. The direct progression of fibrosis over time was defined as the difference in the stages of fibrosis between the first and the second biopsy, i.e. progression during the interval of years between the two biopsies, with the final result expressed in fibrosis units per year (Table 5).

Peer review

The scientific conclusions are reliable and valuable for practical medicine. The references are appropriate, relevant and updated. The study is of particular interest to the practical medicine.

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T cell responses to hepatitis B surface antigen are detectable in non-vaccinated individuals

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after the third vaccination. Surprisingly, these individuals showed response even before the first vaccination. T cell response to control antigens and mitogens was similar in all groups.

CONCLUSION: Our data suggest that there is no general immune deficiency in non-/low-responders. Thus, we hypothesize that the induction of anti-HBsAg responses by vaccination is significantly dependent on the pre-existing T cell repertoire against the specific antigen rather than the presence of a general T cell defect.

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Key words: Hepatitis B; T cell; Non-responder; Vaccination

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Abstract

AIM: To evaluate, whether humoral hepatitis-B-vaccine non-responders also fail to mount a T cell response and to compare these results to normal vaccines.

METHODS: Forty-seven health care employees were enrolled in this study including all available non-responders ($n = 13$) with an anti-HBsAg titer < 10 kU/L and all available low-responders ($n = 12$) with an anti-HBsAg titer < 100 kU/L. Also, 12 consecutive anti-HBsAg negative pre-vaccination subjects were enrolled as well as 10 subjects (+7 from the vaccinated group) with titers > 1000 kU/L as controls. PBMC from all subjects were analyzed by IFN- γ and IL-4 ELISPOT assays for the presence of hepatitis B surface antigen (HBsAg) reactive T cells.

RESULTS: Non-responders and low-responders had no or only very limited T cell responses, respectively. Individuals responding to vaccination with the induction of a high anti-HBsAg titer showed a strong T cell response

INTRODUCTION

Worldwide, hepatitis B virus (HBV) infections are a growing problem with a high prevalence of over 8% hepatitis B surface antigen (HBsAg) positive individuals in Africa, South America, parts of Eastern Europe, South Asia, and Canada^[1]. It is estimated that approximately 350 million people are chronic carriers of HBV with 1-1.5 million dying from liver cirrhosis and primary liver cancer^[2]. Nowadays, protective repeated vaccinations with recombinant HBsAg are not only recommended to all health care workers and travellers, but have recently been included in the childhood and adolescence immunization schedule. Hepatitis B vaccinations prevent HBV infections as well as its complications in most of the vaccines^[3,4]. However, 5% to 10% fail to produce protective anti-HBsAg titers after three vaccinations irrespective of the source of the antigen, which is a problem not only for health care workers, and represents a major medical as well as economic challenge^[5-7]. Low- (HBsAg titer 10-99 kU/L) or

non-responsiveness (HBsAg titer < 10 kU/L) to vaccination are associated with certain human leukocyte antigen (HLA)-class II alleles. DRB1*0301, DRB1*0701, and DQB1*0201^[5,8,9] were shown to have a higher prevalence in non-responders, whereas other antigens (DRB1*0101, *1301, *1501, and DPB1*0401) seem to mediate strong immune responses^[9-12]. Higher age, obesity, male gender, smoking, and chronic dialysis are risk factors for a non-/low-responsiveness^[8,9,13-15].

Monitoring of vaccination efficacy is currently performed by measurements of humoral immune responses (antibody titers) using ELISA assays, which does only indirectly reflect antigen-specific T cell responses. However, the T cell response plays an important role in the immune defence against viral infections. Patients with defects in T-cell function or repertoire such as human immunodeficiency virus (HIV) or transplant patients often suffer from opportunistic infections with viruses including cytomegalovirus (CMV), Epstein-Barr virus (EBV), herpes simplex viruses (HSV), and varicella zoster virus (VZV). It remains unclear, if vaccines with no or low anti-HBsAg titer similarly have a compromised T cell response to HBsAg^[16-18]. Thus, it remains an open question, whether a B cell non-responder is also a T cell non-responder. To clarify this issue, we initiated a study to analyze whether HBsAg non- or low-responders are able to mount a significant T cell response against the HBsAg and compared them to "normal" vaccinees.

MATERIALS AND METHODS

Healthy individuals included in the study

Forty-seven healthy employees of the University of Cologne (median age 32 years, range: 19-61 years, 21 male, 35 female) were enrolled in this study. These 47 employees including 12 consecutive health care employees, who received the first hepatitis B vaccine, were included as well as 10 vaccinated subjects with an anti-HBsAg titer > 1000 U/L. Documented non- and low-responders were contacted and asked to participate in the study, of whom 25 agreed. Of these 25, 13 individuals were non-responders as defined by an anti-HBsAg titer of 0-9 IU/L after the third of three vaccinations and 12 were low-responders (titer 10-99 IU/L). In this study, 3 different groups were analyzed. Non-responders ($n = 13$, titer 0-9 U/L), low-responders ($n = 12$, titer 10-99 U/L), vaccinees before the first and after the third vaccination ($n = 12$), and high-responders. The last group comprises 10 selected individuals with an anti-HBs titer > 1000 U/L plus 7 vaccinees after the third vaccine with a titer > 1000 U/L (total $n = 17$). Therefore, the patient number ($n = 47$) and the subjects analyzed in the 3 groups ($n = 54$) do not match.

Cell separation and ELISPOT analysis

PBMC were isolated by Ficoll density centrifugation from 15 mL of blood and stored in liquid nitrogen until performance of ELISPOT assays. Membrane-bottomed 96-well plates (MAHA, Millipore) were coated overnight with 50 μ L of anti-IFN- γ or anti-IL-4 antibodies (Hölzel, Cologne, Germany) at a concentration of 10 mg/L car-

bonate coating buffer (0.1 mol/L Na₂CO₃, 0.1 mol/L NaHCO₃, pH 9.6) at 4°C. Plates were washed three times with RPMI 1640 and incubated with CellGenix medium (CellGenix, Freiburg, Germany) supplemented with 100 mL/L fetal calf serum (FCS) for 1 h at 37°C. Triplets of 2×10^5 PBMC in 100 μ L CellGenix medium containing Glutamax I (Gibco BRL, Karlsruhe, Germany) were added per well and incubated with medium only, 5 mg/L yeast-derived HBsAg (subtype adw, Biotrend, Cologne, Germany), 5 LF/mL tetanus toxoid, or 10 mg/L pokeweed mitogen (PWM) at 37°C and 5% CO₂. After 72 h, plates were extensively washed with PBS/0.05% Tween and incubated with 100 μ L/well of 10 mg/L biotinylated anti-IFN- γ or anti-IL-4 antibodies (Hölzel, Cologne, Germany) for 2 h at 37°C and 5% CO₂. After washing with PBS, plates were incubated with 100 μ L/well of 1:2000 diluted streptavidin-ALP for 1 h at 37°C and 5% CO₂. Development of spots was performed with 50 μ L/well of chromogenic alkaline phosphatase substrate (BCIP/NBT, Sigma Aldrich, Germany). The reaction was terminated after approximately 5 min by rinsing plates with tap water. Spots were counted after drying of plates with an automated AELVIS Plate Elispot reader (AELVIS GmbH, Hannover, Germany). Specific T cell frequencies were calculated by subtracting mean background + 2 times standard deviation of background from counted spots. Therefore, the absolute numbers of spots are relatively low compared to data from other groups.

Statistical analysis

All statistical calculations were performed with the software package SPSS V12.0 for Windows (Chicago, IL). T cell responses between groups were compared by using a 2-sided Student *t*-test. Probabilities $P < 0.05$ were considered as statistically significant.

RESULTS

Subjects with high anti-HBsAg titers show a strong T cell response

All subjects with an anti-HBsAg titer > 1000 IU/L were analyzed for T-cell responses against the HBsAg and tetanus. This group comprised of a total of 17 subjects (6 male, 11 female, including 7 subjects from the vaccinee group) with a median age of 31 years (range: 20-44 years). The average IFN- γ spot count for HBsAg was 5.4 ± 5.1 , the average IL-4 spot count was 1.0 ± 2.1 . The average IFN- γ spot count for tetanus Ag was 9.1 ± 6.2 , the average IL-4 spot count was 3.0 ± 3.7 .

Non-/low responders lack a sufficient T cell response against HBsAg

Twenty-five subjects had an anti-HBsAg titer < 100 IU/L after at least 3 vaccinations, of whom 13 were non-responders (anti-HBsAg titer of 0 IU/L) and 12 were low-responders (mean anti-HBsAg titer 36 ± 16 IU/L). The median age of non-responders and low-responders was 40 years (range: 20-57 years) and 32 years (range: 19-61 years), respectively. Of the 13 non-responders, 4 were male, 9 female, of the 12 low-responders 4 were male, 8

Table 1 Number of spots in IFN- γ and IL-4 ELISPOT assays for all individuals (*n*)

	<i>n</i>	Mean anti-HBsAg titer (IU/L)	IFN- γ response (# of spots)		IL-4 response (# of spots)	
			HBsAg	TT Ag	HBsAg	TT Ag
Non-responders	13	0 \pm 0	1.0 \pm 1.3	9.1 \pm 4.3 (6)	0.2 \pm 0.3	3.5 \pm 3.9 (6)
Low-responders	12	36 \pm 16	1.7 \pm 2.6	7.8 \pm 6.5 (7)	0.5 \pm 1.0	6.2 \pm 10.1 (4)
High-responders	17	> 1000	5.4 \pm 5.1	9.1 \pm 6.2	1.0 \pm 2.1	1.7 \pm 3.9 (13)
Vaccines before 1st vx	12	0 \pm 0 (9)	4.5 \pm 5.7	11.2 \pm 7.3 (11)	0.3 \pm 0.5 (9)	4.5 \pm 4.9 (5)
Vaccines after 3rd vx	12	10 \times > 1000, 1 \times 211, 1 \times 394	7.1 \pm 6.2	10.0 \pm 8.4	2.7 \pm 5.6	3.0 \pm 4.4 (8)

were female. PBMCs were stimulated with medium only, HBsAg, tetanus Ag, or pokeweed mitogen (PWM) to evaluate T-cell specific cytokine release of IFN- γ and IL-4. When assessing T cell responses against the mitogen PWM, there was no difference between non-responders, low responders, or high responders. A strong cytokine production occurred in all samples after stimulation with PWM (positive control). Similarly, when assessing antigen-specific T cell responses against tetanus Ag, we observed comparable T cell responses in all three groups. Non-responders showed a mean IFN- γ response against tetanus Ag of 9.1 ± 4.3 spots (*n* = 6) and a mean IL-4 response of 3.5 ± 3.9 spots (*n* = 6). Low-responders had an average count of 7.8 ± 6.5 IFN- γ spots (*n* = 7) and 6.5 ± 10.0 IL-4 spots (*n* = 4). For high-responders, T cell response against tetanus Ag was 9.1 ± 6.2 IFN- γ spots (*n* = 11) and 4.5 ± 4.9 IL-4 spots (*n* = 5) before vaccination and 10.0 ± 8.4 IFN- γ spots (*n* = 17) and 1.7 ± 3.9 IL-4 spots (*n* = 13, Table 1).

In contrast, when assessing responses to the HBsAg, we observed clear differences between the three groups. Non-responders had a mean anti-HBsAg IFN- γ T cell response of 1.0 ± 1.3 spots and a mean IL-4 T cell response of 0.2 ± 0.3 spots. Low-responders showed a slightly higher T cell response with mean IFN- γ responses of 1.7 ± 2.6 spots and a mean IL-4 T cell response of 0.5 ± 1.0 spots) compared to high-responders with an average IFN- γ spot count for HBsAg of 5.4 ± 5.1 , and an average IL-4 spot count of 1.0 ± 2.1 .

Taken together, although all individuals tested showed strong responses to the mitogen PWM and significant responses to tetanus Ag, there was a clear difference between non-responders and high responders (*P* = 0.006 for IFN- γ results, *P* = ns for IL-4) with low-responders showing intermediate values. These data suggest that antibody titers indeed correlate with T cell responses as assessed by ELISPOT analysis.

T cell responses against HBsAg are measurable in vaccines before the first immunization

To further corroborate the above findings, we assessed T cell responses and anti-HBsAg titers before the first and after the third vaccination in 12 individuals. The median age was 31 years (range: 23-50 years), 3 subjects were male, 9 were female. All had a negative anti-HBsAg titer before the first and an anti-HBsAg titer > 200 IU/L after the third vaccination suggesting all to be within the intermediate or high-titer group. The average T cell response against HBsAg was 4.5 ± 5.7 IFN- γ spots and 0.3 ± 0.5 IL-4 spots

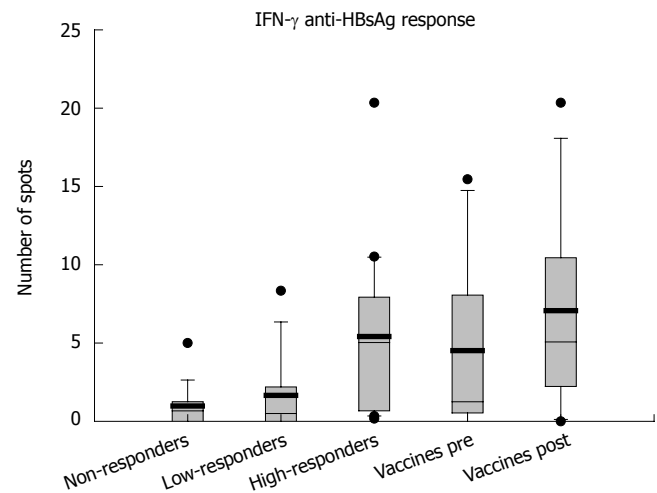


Figure 1 Box and whisker plots of number of IFN- γ spots from ELISPOT assays of PBMC stimulated with HBsAg. The black dots show the outliers.

before the first vaccination and 7.1 ± 6.2 IFN- γ spots and 2.7 ± 5.6 IL-4 spots after the third vaccination, respectively. The difference between pre- and post-vaccination anti-HBsAg response was not significant in a Student *t*-test (*P* = 0.08 for IFN- γ , *P* = 0.10 for IL-4, Table 1 and Figure 1).

DISCUSSION

Approximately 5% to 10% of the healthy population fails to mount a protective anti-HBsAg antibody titer after three vaccinations. However, it has not been evaluated, whether patients with a low antibody titer are also incapable of producing an HBsAg specific T cell response. Our data clearly show that humoral non- and low-responders have no or only a limited T cell repertoire reacting to HBsAg compared to responders. Only 1 out of 13 non-responders and 2 out of 12 low-responders had (IFN- γ) T-cell responses that were equal to or exceeded the average T cell response of the high-responder group. Two other studies have reported decreased IL-2, IFN- γ , and IL-10 cytokine production in ELISA assays of non-responders to HBsAg stimulation and conclude that these subjects may have a defect in either the primary HBsAg-specific T cell repertoire or antigen presentation^[16,17]. Our ELISPOT data clearly indicate that non- and low-responders do not have a general immune defect as their T cell response to control antigens (e.g tetanus toxoid) is comparable to that of “normal” vaccines. Also, their lymphocytes were capable of strong cytokine secretion after PWM stimulation.

So what prevents a non-responder from developing a protective HBsAg titer? As non-responsiveness to HBsAg is associated with certain HLA-haplotypes, it has been hypothesized that antigen presenting cells of non-responders are unable to adequately present this specific antigen^[19], although a recently published trial showed that HLA-DR0301 non-responders are not deficient in their HBsAg-presentation and do not lack B7 co-stimulatory molecules^[20]. Other studies suggested that non-responsiveness was caused by the presence of suppressor T cells^[21,22] or the absence of the Th1 cells or cells with TCR specificity for HBsAg^[23]. Salazar *et al*^[24] reported that non-responders show a defect in HBsAg reactive CD4⁺ helper T cells. Our data extend these hypotheses suggesting that a preexisting T cell repertoire exists in the majority of vaccines that may be critical for strong post vaccination T cell responses. Except for 2 out of 12 individuals, all vaccines had a (IFN- γ) T cell response before vaccination. In contrast, non-/low-responders lacked a significant T cell response. Thus, we hypothesize that a pre-existing immunologic T cell cross-reactivity against the HBsAg is necessary in order to respond to vaccinations.

This cross-reactivity is probably triggered by a common infectious agent, which is processed by antigen presenting cells to peptides similar in structure to parts of the HBsAg. In certain HLA-haplotypes MHC/peptide complexes do or do not induce crossreactive effector or regulatory T cells. This could be the explanation, why non-/low-responsiveness is linked to certain MHC/HLA-types. What we do not know, is whether this cross-reactive T cell response alone yields any protection against a HBV infection. But because HBsAg vaccinations are a very effective measure against a new infection^[25,26], it seems that a concurrent B cell response (*i.e.* protective anti-HBsAg titer) is primarily necessary to prevent HBV replication.

In our trial, IFN- γ was used to study TH1 response and IL-4 for TH2 response. However, the number of spots were relatively low, which has been reported before^[27]. It is especially surprising that the TH2 response was very low in our responders although the hepatitis B vaccine is considered to be an immunization primarily based on a strong TH2/B-cell response. However, our data are in good concordance with other studies demonstrating a dominant TH1 cell response after hepatitis B vaccination^[27-30]. Bauer *et al* recently showed that 15 hepatitis B individuals, who had been successfully vaccinated, but had lost anti-HBs titers were able to mount a significant TH1 response similar to our responders^[27]. This again shows that non- and low-responders significantly differ from responders, even if the latter lose their anti-HBsAg titers.

In light of the burden of HBV infection several strategies are currently under study to increase the efficacy of HBsAg vaccination, e.g. by applying double vaccination dose^[31], using intradermal boosters^[32-34], or adding adjuvants such as monophosphoryl lipid A (MPLA) or influenza vaccine^[35,36]. Although these measures were more effective than the yeast derived hepatitis B vaccine alone, not all non-responders showed seroconversion. Currently, early studies using immunostimulatory CpG oligodeoxynucleotides (CpG ODN) suggest a breakthrough in hepatitis B prevention. Not only do vaccines develop considerably higher titers after

three vaccinations with this new combination vaccine, but most of them already mount protective titers after the first vaccination compared to none of the controls immunized with HBsAg alone^[37]. It will be interesting to study whether the seroconversion after HBV-CpG ODN vaccination is also accompanied by the induction of T cell responses as shown here and whether previous non-responders will now mount comparable T cell responses as a result of vaccination. As the safety of a CpG-ODN vaccine is still to be evaluated, a possible future strategy could be to identify possible non-/low-responders with an ELISPOT assay and administer CpG-ODN admixed vaccine for these individuals. The majority of people could still be vaccinated with the common hepatitis B vaccine.

COMMENTS

Background

Non-responsiveness to hepatitis B vaccine is a problem often experienced by professional health-care workers. It is believed that non-responders lack certain properties in their immune system or are even immunocompromised.

Innovations and breakthroughs

Here, we find that all subjects responding to the hepatitis B vaccine already had a T-cell response against the hepatitis Bs antigen before their first vaccination. We hypothesize that the induction of anti-HBsAg responses is dependent on the pre-existing T-cell repertoire against the specific antigen, which may be expanded by the cross-reaction to a ubiquitous antigen.

Applications

By new adjuvants as CpG-ODN, it may be possible in the future to induce a sufficient anti-HBs antigen response in all vaccines.

Terminology

Hepatitis Bs Ag is the hepatitis B surface antigen (HBsAg). Antibody titers against this antigen are an indicator of a good immune response against the pathogen.

Peer review

In this well written article authors find that all subjects responding to the hepatitis B vaccine already had a T-cell response against the hepatitis Bs antigen before their first vaccination, so they hypothesize that the induction of anti HBsAg responses by vaccination is significantly dependent on the pre-existing T-cell repertoire against the specific antigen rather than the presence of a general T-cell defect, which timely contribute to us.

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RAPID COMMUNICATION

Alteration of sister chromatid exchange frequencies in gastric cancer and chronic atrophic gastritis patients with and without *H pylori* infection

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Author contributions: Karaman A performed SCE analysis in the lymphocytes of all subjects, analyzed all of the data and wrote the article; Binici DN and Dursun H performed endoscopic operations; Kabalar EM and Kurt A analyzed all biopsy materials. **Correspondence to:** Ali Karaman, MD, Erzurum State Hospital, (Erzurum Numune Hastanesi), Department of Medical Genetics, Erzurum 25240, Turkey. alikaramandr@hotmail.com

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H pylori-negative CAG patients (7.93 ± 0.81 vs 6.92 ± 1.20 , $P < 0.05$).

CONCLUSION: We suggest the increased SCE in patients reflects a genomic instability that may be operative in gastric carcinogenesis.

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Key words: Gastric carcinoma; Chronic atrophic gastritis; Pathogenesis; *Helicobacter pylori* infection; Sister chromatid exchange

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Abstract

AIM: To determine, by counting sister chromatid exchange (SCE) frequencies, whether genetic impairment and DNA damage have an effect on the pathogenesis of gastric cancer (GC).

METHODS: Analysis of SCE is a cytogenetic technique used to show DNA damage as a result of an exchange of DNA fragments between sister chromatids. We analyzed SCE frequency in 24 patients with GC, 26 patients with chronic atrophic gastritis (CAG), and 15 normal controls. The presence of *H pylori* was confirmed by urease test, toluidine-blue stain and hematoxylin-eosin stain.

RESULTS: SCE was significantly increased in *H pylori*-negative GC patients, and in *H pylori*-negative CAG patients compared with controls (7.41 ± 1.36 and 6.92 ± 1.20 , respectively, vs 5.54 ± 0.8 , $P < 0.001$). There was no difference in the SCE frequency between *H pylori*-negative GC patients and *H pylori*-negative CAG patients ($P > 0.05$). On other hand, the SCE frequencies in *H pylori*-positive GC patients were higher than those in *H pylori*-positive CAG patients (9.20 ± 0.94 vs 7.93 ± 0.81 , $P < 0.01$). Furthermore, *H pylori*-positive GC patients had a higher SCE frequency than *H pylori*-negative GC patients (9.20 ± 0.94 vs 7.41 ± 1.36 , $P < 0.001$). Similarly, a significant difference was detected between *H pylori*-positive CAG patients and

Karaman A, Binici DN, Kabalar ME, Dursun H, Kurt A. Alteration of sister chromatid exchange frequencies in gastric cancer and chronic atrophic gastritis patients with and without *H pylori* infection. *World J Gastroenterol* 2008; 14(16): 2534-2539 Available from: URL: <http://www.wjgnet.com/1007-9327/14/2534.asp> DOI: <http://dx.doi.org/10.3748/wjg.14.2534>

INTRODUCTION

Gastric cancer is the second leading cause of cancer death and the fourth most common cancer in terms of new cases worldwide^[1]. The development of gastric cancer in humans has been shown to be a multi-step process, ranging from chronic gastritis to atrophy, intestinal metaplasia, dysplasia and finally, invasive cancer^[2-5].

Multiple genetic and epigenetic alterations in oncogenes, tumor suppressor genes, cell-cycle regulators, cell adhesion molecules, DNA repair genes and genetic instability, as well as telomerase activation, are implicated in the multi-step process of gastric carcinogenesis. p53, a tumor suppressor gene is thought to play a critical role in the multistep process of gastric carcinogenesis^[6-9]. Inactivation of p53 by 17p (p53), loss of heterozygosity (LOH) and mutation seems to be an early event in neoplastic progression in gastric carcinomas, because it develops in diploid cells before aneuploidy and other LOH events involving chromosomes 1, 5, 6, 7, 10, 11 and 12^[10,11].

H pylori is an important human pathogen, responsible for most cases of chronic gastritis, peptic ulcer, gastric cancer and gastric mucosa-associated lymphoid lymphoma^[12-16]. Evidence that it acts as a carcinogen has come mainly from epidemiological studies^[17-19] and animal studies^[20]. The working group of the Agency for Research on Cancer reported in 1994 that *H pylori* is indeed a group-1 carcinogen^[21].

H pylori is a carcinogen in humans, although it is not thought to cause gastric cancer directly. It may, however, provide a suitable environment, by causing chronic gastritis and intestinal metaplasia, for neoplastic changes. *H pylori* infection leads to changes in many factors, such as the vitamin C content of gastric juice, the levels of reactive oxygen metabolites in the tissues and epithelial cell proliferation, which are important in the pathogenesis of gastric cancer^[22].

The sister chromatid exchange (SCE) phenomenon is widely used as a reliable and sensitive indicator of chromosome (DNA) instability, since the SCE patterns can reveal a general genome instability^[23]. Variations in DNA repair mechanisms or detoxifying enzymes have been implicated as causing genetic susceptibility associated with cancer^[24]. SCE in peripheral lymphocytes has been widely used to assess exposure to mutagens and carcinogens^[25-27]. The SCE frequency was found to be significantly higher in individuals with Werner syndrome, Bloom's syndrome, and myelodysplastic disease than in their control groups. These diseases are known to be associated with genomic instability^[28,29].

Several groups of investigators have suggested active oxygen species may be implicated in the production of high basal SCE frequencies in chromosome instability syndrome cells, because oxygen free radicals are thought to be responsible for chromosome damage in these cells^[30]. Oxidative damage to DNA over time can cause changes to both the structure and function of chromosomes. These changes in the genetic code may lead to cancer and other chronic diseases^[31,32]. The mutagenic effects of reactive oxygen species (ROS) have been detected in human lymphocytes by using the SCE technique; elevated ROS in cells can cause an increase in mitotic recombination frequency^[33]. Recently, the genotoxicity of ROS has been well established, and oxidative stress has been shown to cause genomic damage^[34,35].

The aim of this study was to determine, by counting SCE frequencies, whether genetic impairment and DNA damage have an effect on the pathogenesis of GC.

MATERIALS AND METHODS

Patients

This study was conducted between February 2007 and June 2007 in the Erzurum State Hospital. We performed SCE analysis in 24 non-smoking (8 females and 16 males) patients with GC (age, mean \pm SE: 62.2 \pm 5.94 years), 26 non-smoking (7 females and 19 males) patients with CAG (age, mean \pm SE: 54.3 \pm 12.27 years), and 15 healthy, non-smoking (6 females and 9 males) controls (age, mean \pm SE: 51.26 \pm 6.27 years). Nine of the 24 GC patients were infected with *H pylori*. Nine of the 26 CAG

patients infected with *H pylori*. The presence of *H pylori* was confirmed by the urease test, toluidine-blue stain and hematoxylin-eosin stain. The patients were selected from non-smoking and nonalcoholic subjects. None of the subjects had a history of viral infection, bacterial infection or any metabolic diseases. The patients had not been treated with chemotherapy or radiotherapy during the last 4 mo. The patient and control groups were chosen for their similar habits. The hospital Ethical Committee approved the human study. All patients were analyzed prior to treatment.

Sister chromatid exchange analysis

For SCE analysis, 2 mL of heparinized blood was drawn from each individual. Cultures were established by adding 0.5 mL of blood to 5 mL karyotyping medium (Biological Industries, Beit Haemek, Israel) with 2% phytohaemagglutinin M (PHA) (Biological Industries, Beit Haemek, Israel), and incubating for 24 h at 37°C. A 5-bromo-2'-deoxyuridine (BrdU) (Sigma, USA) solution at a final concentration of 5 μ g/mL was added. Lymphocytes were cultured in the dark for 48 h and metaphases were blocked during the last 2 h with colcemid (Biological Industries, Beit Haemek, Israel) at a final concentration of 0.1 μ g/mL. Further processing included hypotonic treatment, fixation, slide preparation and fluorescein plus Giemsa (FPG) staining for the detection of SCE^[36]. Fifty second-division metaphases were scored on coded slides by a single observer as the number of SCEs/cell per subject. The SCE data were analyzed statistically by Student's *t*-test.

RESULTS

The associations of GC and CAG with SCE frequencies in *H pylori*-positive and negative groups are shown in Table 1. According to these results, there was no difference in mean SCE frequency between *H pylori*-negative GC patients and *H pylori*-negative CAG patients (7.41 \pm 1.36 *vs* 6.92 \pm 1.20 per metaphase, respectively; *P* > 0.05); however, the mean SCE frequencies of both patient groups were significantly higher than that of the control group (5.54 \pm 0.8 per metaphase, *P* < 0.001 for both patient groups). On the other hand, the mean SCE frequency of *H pylori*-positive GC patients was significantly higher than that of *H pylori*-positive CAG patients (9.20 \pm 0.94 *vs* 7.93 \pm 0.81 per metaphase, respectively; *P* < 0.01). Furthermore, the mean SCE frequency in *H pylori*-positive GC patients was higher than that in *H pylori*-negative GC patients (9.20 \pm 0.94 *vs* 7.41 \pm 1.36 per metaphase, respectively *P* < 0.001). Similarly, *H pylori*-positive CAG patients had a higher mean SCE frequency than *H pylori*-negative CAG patients (7.93 \pm 0.81 *vs* 6.92 \pm 1.20 per metaphase, respectively *P* < 0.05).

DISCUSSION

Gastric cancer is still a common cause of cancer-related deaths worldwide, despite improved diagnostic and therapeutic implications. Hence, early diagnosis has critical importance. Cancer results from accumulated genetic or

Table 1 SCE frequency in *H pylori*-positive and -negative groups of patients and healthy controls (mean \pm SE)

		Sex F/M	n	Age, yr	Age at diagnosis, yr	SCE
GC Patients	<i>H pylori</i> -positive	3/6	9	53.77 \pm 10.27	53.33 \pm 9.26	9.20 \pm 0.94
	<i>H pylori</i> -negative	5/10	15	63.20 \pm 6.98	63.06 \pm 7.06	7.41 \pm 1.36
CAG Patients	<i>H pylori</i> -positive	2/7	9	51.33 \pm 11.21	50.11 \pm 15.16	7.93 \pm 0.81
	<i>H pylori</i> -negative	5/12	17	57.23 \pm 13.58	56.65 \pm 13.65	6.92 \pm 1.2
Controls		6/9	15	51.26 \pm 6.27		5.54 \pm 0.8

GC: Gastric cancer; CAG: Chronic atrophic gastritis.

epigenetic alteration(s) in a variety of genes that directly or indirectly control cell division, cell differentiation, and cell death^[37]. The development of gastric cancer in humans has been shown to be a multi-step process, ranging from chronic gastritis to atrophy, intestinal metaplasia, dysplasia and finally invasive cancer^[2-5].

Exposure of cells to a variety of genotoxic and cytotoxic agents has the potential to elicit prolonged and dynamic changes that compromise the stability of the cellular genome^[38]. Many of these changes, whether induced directly or indirectly by DNA damage, lead to increases in gene mutation and amplification, reduced cloning efficiency, elevated micronuclei, sister chromatid exchanges, and multiple karyotypic abnormalities^[38].

Cytogenetic tests have been widely used in medicine for the assessment of a causal association between disease and cytogenetic damage. In the present study, we investigated whether cytogenetic abnormalities participate in the pathogenesis of GC. SCE, as an indicator of DNA damage, might reflect an instability of DNA or a deficiency of DNA repair. Therefore, it could be used to investigate any causal association between various diseases and any cytogenetic damage^[39-41].

SCE is known to be increased by exposure to various genotoxic carcinogens^[42] and seems to reflect the repair of DNA lesions by homologous recombination^[43]. Important sources of exposure include diet, general environment, medical exposure to ionizing radiation, and internal generation of genotoxic species. Internal phenomena, such as metabolism, errors of DNA replication, inflammation and oxidative stress, may be of importance. Inflammatory diseases, oxidative stress and radiation exposure have been associated with the generation of clastogenic factors, which may be quite persistent^[44-46] and might play an important role in carcinogenesis.

Numerous studies have clarified the relationship between *H pylori* infection and gastric cancer^[14-16]. Epidemiological studies have shown that *H pylori* infection is an important risk factor in gastric cancer^[22,47]. Several *H pylori* virulence-associated genes have been found in Western populations to be associated with an increased risk of gastric cancer and precancerous lesions^[48]. Studies from Japan have confirmed IL-1 β polymorphisms do contribute to the gastric acid secretory response to *H pylori* infection, and subsequently to clinical sequelae^[49,50]. A polymorphism in the IL-1 β gene cluster, which has both pro-inflammatory and potent acid suppressive effects, is associated with an augmented cytokine response to *H pylori* infection that increases the risk of gastric atrophy, gastric ulcer, and gastric cancer^[3,51].

Tsai *et al*^[52] reported alterations in gene expression associated with cell damage, inflammation, proliferation, apoptosis, and intestinal differentiation in gastric tissues, taking into account *H pylori* status. More changes in gene expression, possibly associated with persistent *H pylori* infection and progression of preneoplasia, were observed in the placebo group. No gene was upregulated over time in tissues from the treatment group. This observation is consistent with current knowledge that *H pylori* infection induces cell hyperproliferation, inflammation, and genomic instability^[53].

The frequency of SCE is increased in patients with carcinoma of cervix uteri, nasopharyngeal carcinoma, prostate carcinoma, ovarian carcinoma, acute leukemia, chronic lymphocytic leukemia and breast cancer^[54-59]. Concerning gastric cancer, in one of the earliest studies SCE was increased to similar levels in patients with GC and those with CAG. However, the mean frequencies of both groups were significantly higher than that of the control group^[60]. Furthermore, Gulten *et al*^[61] reported increased SCE frequencies in a group of gastritis patients infected with *H pylori*.

In our study, we found significantly elevated SCE frequencies in both *H pylori*-negative GC patients and *H pylori*-negative CAG patients compared with controls. However, there was no difference in SCE frequency between *H pylori*-negative GC patients and *H pylori*-negative CAG patients. This result is consistent with the study of Zhou L *et al*^[60]. On the other hand, *H pylori*-positive CAG patients had a higher SCE frequency than *H pylori*-negative CAG patients. This finding is consistent with the study of Gulten *et al*^[61]. Similarly, *H pylori*-positive GC patients had a higher SCE frequency than *H pylori*-negative GC patients. Furthermore, the SCE frequencies in *H pylori*-positive GC patients were higher than those in *H pylori*-positive CAG patients. These findings clearly indicate the significance of simultaneous application of SCE for the screening of high-risk individuals. In addition, the results suggest the genotoxic effect of *H pylori* infection is a risk factor for gastric cancer. Intense *H pylori* infection may contribute more to DNA damage and promote carcinogenesis in patients with gastric cancer. Furthermore, chronic *H pylori* infection is also associated with increased gastric cell turnover, which is probably of importance in malignant transformation^[62,63].

Our study, which showed increased SCE frequencies in the lymphocytes of CAG patients, could support these observations, as the induction of changes in DNA that lead to mutations play a role in carcinogenicity. Establishment of inherited susceptibility factors is

important to recognize individuals at a higher risk of developing gastric cancer, so that they may benefit from early detection and prevention programs.

Recent studies have demonstrated a significantly increased risk for the development of gastric carcinoma in patients with CAG^[3,4,64,65]. Patients with CAG have a markedly increased risk of GC, but the mechanism underlying this increased risk is not well understood. Chronic inflammation has been associated with the development of chromosomal aberrations in both disorders that progress to neoplasia, such as ulcerative colitis^[66], and Barrett's esophagus^[67]. Our results confirm and extend these findings to patients with CAG. Many investigators have demonstrated genomic instability and abnormalities in patients with CAG and GC^[60,61,64,65]. Our analysis suggests that chromosomal instability (DNA) is present at very early stages of neoplastic progression in CAG and GC patients. This instability may be permissive for the generation of other genomic aberrations associated with gastric cancer progression.

In conclusion, our results suggest increased chromosomal instability may be associated with the pathogenesis of early gastric cancer. In addition, our findings indicate that the genotoxic potential of *H. pylori* infection is a risk factor for gastric cancer. Thus, SCE is a promising biomarker for assessing the risk of neoplastic progression in gastric carcinoma.

COMMENTS

Background

It is known there is an increased sister chromatid exchange (SCE) frequency in neoplastic diseases. Gastric cancer is still a common cause of cancer-related deaths worldwide, despite improved diagnostic and therapeutic implications. Hence, early diagnosis has critical importance.

Research frontiers

Analysis of SCE is a cytogenetic technique used to show DNA damage as a result of an exchange of DNA fragments between sister chromatids. Therefore, in this study, we aimed to determine, by assessing SCE frequencies, whether genetic impairment and DNA damage have an effect on the pathogenesis of GC.

Innovations and breakthroughs

Our results suggest increased chromosomal instability may be associated with the pathogenesis of early gastric cancer. The identification of increased SCE frequency in patients with gastric lesions may be helpful in the early diagnosis of gastric cancer.

Applications

SCE analysis has come into use as a sensitive means of monitoring the DNA damage. SCE analysis may be used as a marker to estimate the risk of gastric cancer.

Terminology

Sister chromatid exchange (SCE): SCE is known to result from reciprocal DNA interchange in homologous loci of sister chromatids during the replication process.

Peer review

This study indicated genetic impairment and genetic instability may play an important role in gastric cancer. In addition, these findings show the genotoxic potential of *H. pylori* infection is a risk factor for gastric cancer.

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RAPID COMMUNICATION

Diagnostic value of plasminogen activity level in acute mesenteric ischemia

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Abstract

AIM: To investigate the changes in plasminogen activity level during mesenteric ischemia.

METHODS: We performed laparotomy in 90 female Wistar-Albino rats (average weight 230 g). In sham groups (SL) (Groups I and II) the superior mesenteric artery (SMA) and vein (SMV) were explored, but not tied. In SMA groups (Groups III and IV) the SMA was ligated, and in SMV groups (Groups V and VI) the SMV was ligated. On re-laparotomy 2 mL of blood was drawn at 1 h in groups I, III and V, and at 3 h in groups II, IV and VI. Plasminogen levels were assessed and comparisons were made between groups and within each group.

RESULTS: The mean plasminogen activity in the SL group was significantly higher than SMA (25.1 ± 10.8 vs 11.8 ± 4.6 , $P < 0.001$) or SMV (25.1 ± 10.8 vs 13.7 ± 4.4 , $P < 0.001$) groups both at 1 h and at 3 h (29.8 ± 8.9 vs 15.1 ± 5.7 , $P < 0.0001$; 29.8 ± 8.9 vs 14.2 ± 2.9 , $P < 0.0001$). There were no significant differences between the values of SMA and SMV groups at 1 h ($P = 0.28$) and at 3 h ($P = 0.71$). In each group, plasminogen activity levels did not change significantly between the two measurements performed at 1 h and 3 h.

CONCLUSION: We conclude that blood plasminogen activities decrease during early phases of both arterial and venous mesenteric ischemia which may be a useful marker for early diagnosis.

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Key words: Mesenteric ischemia; Necrosis; Activity level

INTRODUCTION

Successful treatment of acute mesenteric ischemia depends on restoration of circulation before the development of intestinal necrosis, and hence on early diagnosis^[1-4]. However, laboratory signs are non-specific even after the development of necrosis and the rate of mortality is still over 50% even in experienced centers^[5-7].

Plasminogen is a protease synthesized in the liver. It is a member of the fibrinolytic system and its plasma level are stable. Plasma plasminogen level is known to decrease in several conditions such as disseminated intravascular coagulation, severe hepatic failure, thrombotic disorders, thrombolytic treatment, and primary and secondary fibrinolysis^[8,9]. Plasminogen is the most important element of the fibrinolytic pathway and our literature search has not revealed any studies examining the change in plasminogen level during acute mesenteric ischemia. Thus, in the present study, the potential use of plasminogen level for the early diagnosis of acute mesenteric ischemia has been investigated.

MATERIALS AND METHODS

The study protocol was approved by the Ethics Committee of Haydarpasa Training and Research Hospital. A total of 90 female Wistar-Albino rats, with an average weight of 230 g (range: 190-270 g), were fed with standard food and water at room temperature, and were allocated into 6 study groups, each having 15 rats.

Surgical procedure

Rats were placed in a bowl containing a cotton pad soaked with diethyl ether for about 3 min and then anesthetized with ketamine 50 mg/kg, IM. After shaving and sterilization of the abdominal wall with povidone

iodine solution, laparotomy was performed through a midline incision. In all groups the superior mesenteric artery (SMA) and vein (SMV) were explored and 3/0 silk suture materials were placed around the vessels. In sham laparotomy groups (Groups I and II) sutures were not tied. In the SMA occlusion groups (Groups III and IV) the SMA was ligated; while in the SMV occlusion groups (Groups V and VI) the SMV was ligated. Abdominal walls were closed with continuous 3/0 silk sutures.

Blood sampling

Exploration and ligation of the artery and the vein were taken as zero time point. Re-laparotomy was performed at 1 h in groups I, III and V, and at 3 h in groups II, IV and VI, and 2 mL of blood was drawn from the heart with a 21G syringe.

Tests and assessments

Two-milliliter blood samples, collected at 1 h and at 3 h, were centrifuged in citrated tubes for 15 min at 4500 r/min. They were then stored at -20°C until being assayed. Plasminogen was assessed as chromogenic enzyme activity by the Streptokinase method by using a Dade Behring Coagulometry. SMA occlusion, SMV occlusion and Sham laparotomy (SL) groups were compared with respect to plasminogen activity levels at 2 time points. Also plasminogen activities at 1 h and at 3 h were compared within each group.

Statistical analysis

The statistical analyses were performed with SPSS (Statistical Package for Social Sciences) for Windows 10.0. Wilcoxon test was used to compare the data obtained at different time points. Kruskal-Wallis H analysis was used to compare three groups and Mann Whitney U Test was used for pair wise comparisons between groups. The results were evaluated at a confidence interval of 95%, and *P* values less than 0.05 were considered statistically significant.

RESULTS

During the first hour of ischemia, the small intestine and right colon were pale and edematous at laparotomy in SMA- and SMV-ligated rats. These structures turned dark red and/or purple by the end of the third hour. At 1 h, the mean plasminogen activity in the SL group was significantly higher compared to the SMA or SMV occlusion groups (25.1 ± 10.8 vs 11.8 ± 4.6 or 13.7 ± 4.4 , $P < 0.001$). Although plasminogen activity levels in the SMA occlusion group were lower than the SMV occlusion group, the difference between the two was not statistically significant. Also, at 3 h, plasminogen activity in the SL group was significantly higher compared to the SMA and SMV occlusion groups (29.8 ± 8.9 vs 15.1 ± 5.7 or 14.2 ± 2.9 , $P < 0.0001$). Plasminogen activities in the SMA occlusion group were higher than those in the SMV occlusion group with no significant difference.

In each group, plasminogen activity levels did not change significantly between the two measurements performed at 1 and 3 h. Plasminogen activity levels within and between groups are presented in the Table 1.

Table 1 Plasminogen activity levels (%) of groups at two time points

Time points	Sham group	SMA occlusion group	SMV occlusion group	<i>P</i> value
1st h	25.1 ± 10.8	11.8 ± 4.6	13.7 ± 4.4	$P < 0.001$
3rd h	29.8 ± 8.9	15.1 ± 5.7	14.2 ± 2.9	$P < 0.0001$
<i>P</i> values	$P = 0.21$	$P = 0.088$	$P = 0.71$	

SMA: Superior mesenteric artery; SMV: Superior mesenteric vein.

DISCUSSION

Both metabolic and morphologic changes are observed during mesenteric ischemia. As reported by Brown *et al*, structural changes start to occur within the first 10 min of ischemia^[10]. It has also been reported that within 30 min of ischemia a remarkable amount of fluid accumulates, both in the basement membrane and between cells resulting in paleness of villi, and also inflammatory cells accumulate^[11,12]. Thus, laboratory markers with high sensitivity and specificity that can be utilized 1 to 2 h prior to the occurrence of irreversible changes would be beneficial in the early diagnosis of acute mesenteric ischemia.

Most of the previous studies examining intracellular enzymes released from ischemic bowel failed to identify any marker sensitive or specific to disease for early diagnosis^[13-15]. The most likely explanation for the failure to identify such an early marker is that these enzymes usually appear in the blood only after irreversible ischemia has occurred in the bowel and that they are taken to the liver through the portal system to be metabolized. Murray *et al*^[16] demonstrated an increase in the blood levels of D(-)-lactate, a product of bacterial metabolism, 2 h following superior mesenteric artery occlusion. However, D(-)-lactate is also known to be produced in increased amounts in several conditions associated with bacterial proliferation, such as non-ischemic obstruction, intestinal perforation and peritonitis. So, D(-)-lactate does not seem to be specific for this disease.

Inorganic phosphate, which is the most important anion at the intracellular space, has also been investigated in models of mesenteric ischemia^[17-20]. A significant rise in serum phosphate level occurred after 4 h of ischemia, which was not helpful for an early diagnosis^[19]. Regional intestinal ischemia is reported to be detected and monitored by means of a microdialysis catheter placed in the peritoneal cavity or the bowel lumen by measuring lactate, pyruvate, glycerol and glucose levels^[21].

Plasma markers of coagulation and fibrinolysis such as fibrinogen, soluble fibrin, D-Dimer, tissue plasminogen activator/plasminogen activator inhibitor complex levels, have previously been studied in intestinal ischemia^[22-26]. However, the change in plasminogen levels, a fibrinolytic marker, has not been studied during acute mesenteric ischemia. In the present study, the role of plasminogen as a novel and specific marker for the early diagnosis of mesenteric ischemia was investigated.

Plasminogen is a single-chain glycoprotein that is produced in the liver and found in plasma under normal conditions; it has a half-life of 2.2 d. Average plasma level

is 21 mg/dL and its enzymatic activity ranges between 75% and 150% in humans. The level of plasminogen activity in rats changes with age and sex of the animal, but it is much lower than those in humans. The parameters we measured in this study are comparable with those in the literature^[26-29]. Plasminogen binds to fibrin as soon as a fibrin clot forms. That is, the fibrinolytic system is activated as coagulation continues. Plasminogen is converted to active plasmin by tissue plasminogen activator (t-PA), which is present in many tissues as well as in plasma and endothelial cells^[30-31]. During intestinal ischemia coagulation and fibrinolysis are activated. Fibrin production is coupled with the conversion of plasminogen to plasmin by t-PA released from hypoxic intestinal endothelium^[20].

In this rat model of acute mesenteric ischemia produced by arterial and venous occlusion, we measured blood plasminogen activity levels after 1 h and 3 h of ischemia and compared the plasminogen activities within and between groups. After 1 h of ischemia, a significantly lower activity of plasminogen was observed in both arterial and venous occlusion groups compared to the sham group, and the difference was maintained up to 3 h of ischemia. No significant differences between plasminogen activity levels after 1 h and 3 h of ischemia were present within the groups. The decrease in plasminogen levels may be attributed to the increased consumption of plasminogen by conversion to plasmin, due to the increased t-PA activity and fibrinolysis.

These data suggest that blood plasminogen activities are decreased during the early phase of both arterial and venous mesenteric ischemia, and plasminogen activity levels may be a useful marker for the early diagnosis of this disease. However, more research is required to exclude other possible causes of the fall in plasminogen activities.

COMMENTS

Background

Acute mesenteric ischemia has no specific marker for early diagnosis and thus, it is mortal in half of the cases. In this study, we investigated significance of changes in plasma plasminogen activity level during mesenteric ischemia.

Research frontiers

In this study we found a significant reduction in plasminogen activity level during early phases of both venous and arterial mesenteric ischemia. However, we need more research to understand its value in human mesenteric ischemia and also we should find equipment in laboratories for urgent plasminogen level determination so that the test could be helpful before bowel necrosis developed.

Innovations and breakthroughs

Several parameters have been studied for early diagnosis of mesenteric ischemia but none of them are found to be valuable. As plasminogen is a member of the fibrinolytic pathway we thought its plasma level could change in the condition and this change could be diagnostic.

Applications

Plasminogen level determination may be helpful in early diagnosis of mesenteric ischemia before bowel necrosis develops and we can manage the patient early preventing mortality.

Terminology

Mesenteric ischemia is an acute condition resulting from occlusion of either mesenteric artery or vein. It is recommended early management is necessary

because after the development of bowel necrosis there is not much to do for the recovery of the condition. Plasminogen is a protein synthesized in liver which is known as a member of the fibrinolytic pathway.

Peer review

The research is very interesting since the author's demonstrated that blood plasminogen activities decrease during early phases of both arterial and venous mesenteric ischemia, which may be a useful marker for early diagnosis. It appears that many potential cases could be lost or misrepresented by the range on values in normal and abnormal groups.

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RAPID COMMUNICATION

Use of infliximab in the prevention and delay of colectomy in severe steroid dependant and refractory ulcerative colitis

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Abstract

AIM: To determine if infliximab can prevent or delay surgery in refractory ulcerative colitis (UC).

METHODS: UC patients who failed to have their disease controlled with conventional therapies and were to undergo colectomy if infliximab failed to induce a clinical improvement were reviewed. Patients were primarily treated with a single 5 mg/kg infliximab dose. The Colitis Activity Index (CAI) was used to determine response and remission. Data of 8 wk response and colectomy rates at 6 mo and 12 mo were collected.

RESULTS: Fifteen patients were included, 7 with UC unresponsive or intolerant to IV hydrocortisone, and 8 with active disease despite oral steroids (all but one with therapeutic dosage and duration of immunomodulation). All the IV hydrocortisone-resistant/intolerant patients had been on azathioprine/6-MP < 8 wk. At 8 wk, infliximab induced a response in 86.7% (13/15) with 40% in remission (6/15). Within 6 mo of treatment 26.7% (4/15) had undergone colectomy and surgery was avoided in 46.6% (7/15) at 12 mo. The colectomy rate at 12 mo in those on immunomodulatory therapy < 8 wk at time of infliximab was 12.5% (1/8) compared with 100% (7/7) in patients who were on long-term maintenance immunomodulators ($P < 0.02$).

CONCLUSION: Infliximab prevented colectomy due to active disease in immunomodulatory-naïve, refractory UC patients comparable to the use of Cyclosporine. In patients, however, on effective dosage and duration of immunomodulation at time of infliximab therapy colectomy was not avoided.

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INTRODUCTION

Ulcerative colitis (UC) is characterized by a life-long chronic course with remissions and exacerbations, with 15% of patients having a severe attack requiring hospitalization at some time during their illness. While the management of a severe exacerbation has traditionally been dependent on intravenous steroids, up to 40% of patients fail to respond resulting in colectomy during that admission^[1,2], this rate has remained unchanged over 30 years^[3]. Even in those who respond, 25% become steroid dependent and an additional 20%-30% require colectomy at 12 mo^[4]. Cyclosporine (CsA) has been shown to be an effective rescue therapy in acute UC in up to 80% of patients^[5], but despite this and the use of azathioprine or 6-mercaptopurine (6-MP) as maintenance therapy, over 65% of patients have relapsed and 30% require colectomy within 12 mo^[6-8]. Concerns over the safety profile of CsA, even with low dose CsA (2 mg/kg per day), has also resulted in reluctance for clinicians to use this due to its well documented side effects^[9].

The early inflammatory bowel disease studies investigating infliximab, a chimeric monoclonal antibody to the proinflammatory cytokine tumour necrosis factor alpha (TNF- α), focused on Crohn's disease where it was known that TNF- α was implicated. Initially the role of TNF- α was considered to be less predominant in UC although more recently TNF- α has been shown to play an important role in

the inflammatory process in UC^[10,11]. The initial uncontrolled published studies suggested efficacy in the use of infliximab for acute steroid refractory UC^[12-14], although only two of three small randomised placebo controlled trials supported its role^[15-17]. Two large randomised placebo controlled trials (ACT 1 and ACT 2) recently confirmed the efficacy of infliximab in moderate UC^[18] and a recent meta analysis of randomised studies in UC demonstrated infliximab to be more effective than placebo, with a mean response rate of 68% and a 40% remission rate at 2 wk, giving the patient number needed to treat as 3 to 5^[19]. In clinical practice, however, patients with acute severe UC requiring IV steroids and chronic steroid refractory UC are clearly distinguished but limited data exists on any differences in efficacy of infliximab in long term outcomes in either group^[20,21]. Only the ACT 1 and ACT 2 studies provide data beyond 3 mo in a randomised controlled trial. These subjects achieved a 45% response and 35% remission at 54 wk (ACT 1), but patients received infliximab every 8 wk following a three dose induction at 0 wk, 2 wk and 6 wk and 78% remained on steroids at one year. These data do not reflect current practice in the management of most patients with UC in the majority of countries around the world and there is little data on long-term outcome following a single dose of infliximab in patients with UC not responsive to standard medical therapy.

In this paper we present our clinical experience of using infliximab in the context of patients with both acute severe UC and chronic refractory UC where surgery was the planned outcome if infliximab failed to induce a clinical improvement in either group.

MATERIALS AND METHODS

All subjects were patients at the Fremantle Hospital in the southern metropolitan region of Perth, Australia, which is a 450 bed tertiary institution and is a centre for the management of IBD. All patients receiving infliximab for IBD were considered for treatment only after failure of disease control with conventional therapy including 5-aminosalicylic acids, steroids and immunomodulation and were treated between August 2001 and December 2006. All patients were planned to go to surgery if treatment with infliximab failed. Patients received a single induction dose of 5 mg/kg in all but one case. Prior to the infliximab infusion patients received a 200 mg IV injection of hydrocortisone unless already on IV hydrocortisone. No prophylactic antibiotics were given. Tuberculosis was excluded in all patients both by a normal chest X-ray and a negative Mantoux test or QuantiFERON-TB Gold (QFT-G, Cellectis Limited, Carnegie, Victoria, Australia). Patients' records were reviewed retrospectively and data was collected from the hospital patient IBD database which includes demographic details, disease duration and extent, endoscopy and medication history, dates, doses and indications for therapy, blood results and recorded adverse outcomes and surgery.

Patients were classified as having UC according to the "Montreal Classification" (a modification of the Vienna Classification)^[22]. The diagnosis of IBD had to be definite, and was made in accordance with previously established

Table 1 Medications taken by patients at time of infliximab treatment

Medication	UC patients (n = 15)
5-ASA	
Current	93.3% (14/15)
Intolerant	6.7% (1/15)
Steroids	
IV	40.0% (6/15)
PO	53.3% (8/15)
Intolerant	6.7% (1/15)
Immunomodulators	
AZA/6MP	86.7% (13/15)
< 8 wk	53.3% (8/15)
≥ 8 wk	33.3% (5/15)
Intolerant	13.3% (2/15)
Tacrolimus	6.7% (1/15)
Methotrexate	6.7% (1/15)

international criteria^[23] based upon clinical, endoscopic, histopathological, and radiological findings. The diagnosis of UC was exclusive of infective enterocolitis (excluded by stool microscopy and culture, bacterial and amoebic serology, acid-fast staining of biopsies and mycobacterial cultures), Behcet's disease and microscopic colitis. Demography, disease status, infusion number, response and remission rates and adverse effects were recorded.

A clinical response was defined as a reduction of 4 or more points in the colitis activity index (CAI)^[24] and remission was considered to be a sustained CAI of less than or equal to 4. This was determined at 2-4 wk and again at 8 wk after the initial infusion. For remission to be considered to have occurred, steroids needed to be successfully withdrawn within the 8 wk period following the infliximab infusion without recurrence of symptoms. Time till colectomy and current UC status was determined from review of the patients' records and IBD database.

Adverse effects were analysed. Adverse effects identified as unlikely related, possibly related and probably related to infliximab use. Serious adverse effects are defined as any adverse drug experience occurring that results in death, life-threatening adverse event, persistent or significant disability/incapacity, required in-patient hospitalisation, or prolonged hospitalisation or congenital anomaly or birth defect.

RESULTS

Patient demographics

Fifteen patients (10 male, 5 female) aged 18 years to 59 years (mean age 39 years) received infliximab at 5 mg/kg. Seven patients had acute severe UC at the time of infliximab, six were not responsive to 5 d of IV hydrocortisone and one was steroid intolerant. All seven of these patients had been on concomitant azathioprine/6-MP for < 8 wk. Eight patients had active disease despite oral steroids and therapeutic dosage and duration of immunomodulatory therapy in all but one patient at the time of infliximab. Fourteen patients were receiving 5-ASAs, with one patient intolerant of this therapy (Table 1).

86.7% (13/15) of patients had extensive UC, one patient left-sided disease and one proctitis as defined by the

Table 2 Individual patient data of disease extent, CAI scores pre and post infliximab, steroid usage and therapeutic immunomodulation (therapy > 8 wk at ≥ 1.5 mg/kg of 6-MP) at time of infliximab and time to colectomy

	Sex	Age at diagnosis	Age at infliximab	Disease extent	CAI at infliximab	CAI at 8 wk	On steroids IV or oral	Therapeutic immuno-modulation	Response to infliximab	Time to surgery (mo)
1	F	37	59	Pan Colitis	14	4	Intolerant	No	Complete	
2	M	25	26	Pan Colitis	14	0	IV	No	Complete	
3	F	28	28	Pan Colitis	11	N/A	IV	No	Partial	1
4	F	45	49	Pan Colitis	14	4	IV	No	Complete	
5	F	54	54	Pan Colitis	20	6	IV	No	Partial	
6	M	24	28	Pan Colitis	13	4	IV	No	Complete	
7	F	22	22	Pan Colitis	11	6	IV	No	Partial	
8	M	17	18	Pan Colitis	13	0	Oral	No	Complete	26
9	M	56	58	Proctitis	12	11	Oral	Yes	Nil	8
10	M	54	55	Left sided	15	N/A	Oral	Yes	Nil	< 1
11	M	32	40	Pan Colitis	10	6	Oral	Yes	Partial	12
12	M	25	31	Pan Colitis	16	3 (on steroids)	Oral	Yes	Partial	5
13	M	27	28	Pan Colitis	14	9	Oral	Yes	Partial	9
14	M	32	38	Pan Colitis	8	3 (on steroids)	Oral	Yes	Partial	9
15	M	56	40	Pan Colitis	13	1	Oral	Yes	Complete	6

Table 3 Remission and response rates at 8 wk to infliximab in the UC patients and colectomy rates at 6 mo and 12 mo

Disease type	Response wk 8	Remission wk 8	Colectomy ≤ 6 mo	Colectomy ≤ 12 mo
E1 - proctitis	0% (0/1)	0% (0/1)	0% (0/1)	100% (1/1)
E2 - left sided	0% (0/1)	0% (0/1)	100% (1/1)	100% (1/1)
E3 - extensive	100% (13/13)	46.20% (6/13)	23.10% (3/13)	46.10% (6/13)
Total	86.70% (13/15)	40.00% (6/15)	26.70% (4/15)	53.30% (8/15)

Table 4 Colectomy rates at 6 mo and 12 mo compared to clinical response to infliximab

Response	No surgery at 12 mo	Colectomy ≤ 6 mo	Colectomy ≤ 12 mo
No response	0% (0/3)	50% (1/2)	100% (2/2)
Partial response	28.60% (2/7)	28.60% (2/7)	71.40% (5/7)
Complete response	83.30% (5/6)	16.70% (1/6)	20% (1/5)
Total	46.70% (7/15)	26.70% (3/16)	57.10% (8/14)

Montreal classification^[22]. The average CAI was 13 (SEM ± 2.8 , range 8-20; Table 2) and 84.6% of patients had a raised CRP (Median 42 mg/L, range 8-110 mg/L) pre infliximab.

Efficacy at 8 wk

At 8 wk 86.7% (13/15) of patients had responded to infliximab with 40% (6/15) in remission (Tables 2 and 3) as determined by the CAI. All those patients considered to be in remission also had had their steroids withdrawn by the 8 wk assessment. Of the responders, all had extensive colitis, while the two non-responders either had proctitis or left-sided UC (Tables 2 and 3). The median CRP at 8 wk was 21.4 mg/L (range < 1-88 mg/L).

Efficacy at 6 mo

At 6 mo 26.7% (4/15) of patients had undergone colectomy, 2 within the first mo of infliximab and 1 at 5 mo and 1 at 6 mo (Tables 3 and 4). Three of these patients were on therapeutic dosages and durations of immunomodulators at the time of the infliximab therapy (defined as > 8 wk duration of 6-Mercaptopurine at ≥ 1.5 mg/kg). One of the two patients who had a colectomy within 1 mo of infliximab was not on a therapeutic duration of immunomodulation, but had no active UC endoscopically post infliximab. She, however, suffered from recurrent massive

acute colonic bleeding that required hospitalization on 2 separate occasions that included blood transfusions. This patient chose to undergo colectomy. Pathology of the surgical specimen identified mild active chronic colitis with no cause of the massive colonic bleeding identified. Despite this, surgery resolved the bleeding issue. The other patient had had no response to the infliximab.

The patient who underwent colectomy at 6 mo also had a complete response to infliximab therapy but was intolerant of azathioprine/6-MP, and had suffered from chronic ankle pain that commenced within minutes of the infliximab infusion and remained for the next 6 mo. The patient also developed pneumonia 2 wk after infliximab therapy and when he developed a significant flare of his UC, he decided to undergo surgery rather than further infliximab treatments.

Efficacy at 12 mo

By 12 mo a further 26.7% (4/15) of patients had undergone colectomy giving a 12 mo colectomy rate of 53.3% (8/15). All of these patients were on therapeutic dosages and durations of immunomodulators at the time of the infliximab therapy. Three were on azathioprine/6-MP and one was on oral tacrolimus due to intolerance to azathioprine/6-MP. Of the 4 patients who underwent colectomy within 6 mo to 12 mo 1 was for steroid depen-

dent proctitis. Two patients had colectomy at nine mo and one at 12 mo for pan colitis. These 3 patients had an initial partial response to infliximab (Tables 3 and 4).

There was a significant difference between the 12 mo colectomy rate for those patients who were on therapeutic dosages and durations of immunomodulators compared with those patients on immunomodulatory therapy for < 8 wk at the time of infliximab therapy [100% (7/7) *vs* 12.5% (1/8), $P < 0.02$].

Long term follow up

Of the seven patients who avoided surgery at 12 mo one patient required colectomy at 26 mo for an acute flare but six remained off steroids and was maintained on azathioprine/6-MP with follow up averaging 25.8 mo (range 12-56 mo).

Side effects

One patient had an adverse side effect to infliximab with the development of arthralgia in his ankle within minutes of initiation of the infliximab infusion, which persisted for 6 mo. The same patient had a significant adverse event with the development of community acquired pneumonia 2 wk post infusion which was treated with standard antibiotic therapy with no long term sequelae. No post operative complications were recorded.

DISCUSSION

Infliximab has become an accepted treatment option following recent data on its efficacy in moderate to severe UC with a 35%-40% remission rate at 8 wk^[19]. Five randomised double blind studies have compared infliximab with placebo for the treatment of UC^[15-18] with two further randomised studies comparing infliximab with steroids^[25,26]. Despite significant heterogeneity in the study populations and trial designs used, all but one study demonstrated a significant improvement in the disease treated with infliximab compared to placebo^[16]. Apart from the ACT 1 and ACT 2 studies, however, all the studies have had limited follow-up of 3 mo or less and have not used maintenance infliximab therapy. The remission rates in ACT 1 using infliximab at 5 mg/kg were 38.8%, 33.9% and 34.7% at wk 8, 30 and 54 respectively; with 78% of patients still requiring steroids at 54 wk despite 8 wk infliximab. Given the financial implications of regular maintenance infliximab therapy and the lack of data comparing induction therapy alone with induction plus maintenance therapy in longer-term outcomes in UC our study retrospectively analysed the outcomes of all UC patients at our institution who were to undergo colectomy if infliximab therapy did not induce a clinical improvement. All patients who received infliximab had confirmed ongoing active disease despite steroids.

The 86% response rate and 40% remission rate at 8 wk in our patients is comparable with previous published studies. At 6 mo only 4 of 15 patients had required colectomy, 1 of whom was a complete responder at 8 wk, but who was intolerant of 6-MP and who declined further infliximab treatment following a flare due to its side effects. Another patient chose to undergo colectomy within 1 mo of infliximab for recurrent massive PR bleeding for which no

cause could be identified but endoscopically her UC was in remission. By 12 mo 53% (8 of 15 patients) had undergone colectomy. Five of the seven patients who avoided surgery were in remission at 8 wk following a single infliximab infusion and the remaining 2 had an initial response but not remission. Six of the seven patients who avoided surgery had acute severe UC unresponsive to IV hydrocortisone and had been on 6-MP for less than 8 wk at the time of infliximab. The seventh patient was on high dose oral steroids and had also been on 6-MP for less than 8 wk. The efficacy of infliximab in these patients with more acute disease in our institution is in keeping with higher response rates to infliximab in studies of steroid refractory UC when compared with steroid dependent cases^[27], although the data remains conflicting^[20,28]. It would be anticipated, however, that a greater role of TNF- α in severe UC would result in a greater clinical efficacy of infliximab in this setting. The fact that colectomy was prevented at 12 mo in 7 of the 8 patients, (with one patient having surgery but without active UC) who were naive to immunomodulators (*ie* less than 8 wk of therapy), suggests that infliximab could be used as bridging therapy. This induces remission whilst giving more time for azathioprine/6-MP to reach therapeutic effect which can then be used to maintain this without the need for regular maintenance infliximab. A similar phenomenon has recently been reported with the use of CsA in acute severe colitis where the colectomy rate was significantly lower in patients who were commenced on azathioprine concurrently with CsA compared with those who were already on azathioprine^[6-8]. In those patients where immunomodulatory therapy has failed, however, regular maintenance therapy with infliximab may be of greater benefit although further studies are required to assess the risks of regular biological therapy versus colectomy in this group of patients.

Our data suggest that at 12 mo the outcome following a single induction dose of infliximab is similar to that seen in the ACT studies, without the need for regular maintenance infusions, provided immunomodulatory therapy can be commenced and continued. This is supported by a recent paper from Oxford assessing their use of single dose infliximab in UC^[20]. This has significant cost implications since the direct cost saving of single dose infliximab therapy with maintenance immunomodulators compared to maintenance infliximab therapy over one year would be €15 000 (\$20 000 US) per patient. Whilst the limitations of this study, being a small, uncontrolled retrospective analysis, prevent direct comparisons being made, further controlled studies assessing the long-term outcomes of induction therapy without maintenance infliximab are warranted.

Infliximab was well tolerated in all but one patient who developed acute arthralgia, within minutes of the infusion, which lasted 6 mo. This patient also developed community-acquired pneumonia 2 wk after infliximab, which responded to standard antibiotic therapy. Despite complete remission at 8 wk he subsequently flared at 5 mo (having been intolerant of 6-MP) and went to colectomy after declining further infliximab therapy. The side effect profile of infliximab is highly relevant when considering rescue therapy for severe UC as both CsA and infliximab have

similar response rates, although there is currently no randomised trial comparing their efficacy and safety in acute severe UC. Whilst the retrospective nature of our data analysis may not have detected mild adverse events associated with infliximab our data are consistent with published studies which demonstrate an adverse side effect profile in UC similar to that seen in Crohn's disease^[29]. This may favour the use of infliximab over cyclosporine as a rescue therapy, as there has been reluctance in some countries to use cyclosporine, even at 2 mg/kg, due to the risks of hypertension, seizures and nephrotoxicity^[30].

In conclusion, infliximab induced a rapid response in over 80% of UC patients refractory to standard therapy with a 40% remission rate at 8 wk. There was avoidance of colectomy in 46% of all patients at 12 mo, without the need for regular infliximab maintenance therapy. There was, however, a significant difference in the 12 mo colectomy rate for those patients who were on therapeutic dosages and durations of immunomodulators compared with those patients on immunomodulatory therapy for < 8 wk at the time of infliximab therapy with 87.5% of these patients avoiding colectomy.

COMMENTS

Background

Corticosteroids have long been the predominant therapy for the induction of remission in severe ulcerative colitis (UC) with immunomodulators being efficacious in maintaining remission. However, over 30% fail to respond or lose response to therapy and require surgery. Cyclosporine has been used as a salvage therapy but 30% proceed to colectomy within 12 mo. The introduction of new biological agents such as infliximab have been shown to be efficacious in acute severe UC but data on long term follow up is limited.

Research frontiers

Infliximab has recently become an accepted treatment option in moderate to severe UC but the only prospective randomised long term data is in patients on maintenance infliximab for mild to moderate UC. This study was aimed at assessing the long term efficacy, safety profile and need for colectomy, following induction therapy with infliximab, without maintenance infliximab, in patients with UC who had failed standard medical therapy.

Innovations and breakthroughs

A single dose of infliximab had greater efficacy in patients with severe acute disease in our institution rather than in those with chronic UC who were flaring despite current treatment. This is in keeping with higher response rates to infliximab in studies of steroid refractory UC when compared with steroid dependent cases and may reflect a greater role of TNF- α in early disease.

Applications

Infliximab induction therapy is safe and appropriate in acute UC patients failing standard medical therapy but response rates appear poor in those with chronic refractory disease. A head to head randomised controlled trial of cyclosporine versus infliximab for the induction of remission in acute severe UC is urgently needed.

Terminology

Infliximab is a chimeric monoclonal IgG1 antibody directed against tumour necrosis factor- α (TNF- α) which is able to almost completely neutralize its biological activity.

Peer review

A single dose of infliximab had greater efficacy in patients with severe acute disease in this study rather than in those with chronic UC who were flaring despite current treatment. A small but useful retrospective study looking at the treatment

outcomes following a single dose of infliximab as rescue therapy in refractory UC in a clinical practice setting.

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RAPID COMMUNICATION

Treatment of gastric remnant cancer post distal gastrectomy by endoscopic submucosal dissection using an insulation-tipped diathermic knife

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long-term outcome needs to be evaluated in the future.

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Key words: Remnant stomach; Distal gastrectomy; Gastric cancer; Endoscopic mucosal resection; Insulation-tipped diathermic knife

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Abstract

AIM: To evaluate the effectiveness of endoscopic submucosal dissection using an insulation-tipped diathermic knife (IT-ESD) for the treatment of patients with gastric remnant cancer.

METHODS: Thirty-two patients with early gastric cancer in the remnant stomach, who underwent distal gastrectomy due to gastric carcinoma, were treated with endoscopic mucosal resection (EMR) or ESD at Sumitomo Besshi Hospital and Shikoku Cancer Center in the 10-year period from January 1998 to December 2007, including 17 patients treated with IT-ESD. Retrospectively, patient backgrounds, the one-piece resection rate, complete resection (CR) rate, operation time, bleeding rate, and perforation rate were compared between patients treated with conventional EMR and those treated with IT-ESD.

RESULTS: The CR rate (40% in the EMR group *vs* 82% in the IT-ESD group) was significantly higher in the IT-ESD group than in the EMR group; however, the operation time was significantly longer for the IT-ESD group (57.6 ± 31.9 min *vs* 21.1 ± 12.2 min). No significant differences were found in the rate of underlying cardiopulmonary disease (IT-ESD group, 12% *vs* EMR group, 13%), one-piece resection rate (100% *vs* 73%), bleeding rate (18% *vs* 6.7%), and perforation rate (0% *vs* 0%) between the two groups.

CONCLUSION: IT-ESD appears to be an effective treatment for gastric remnant cancer post distal gastrectomy because of its high CR rate. It is useful for histological confirmation of successful treatment. The

Hirasaki S, Kanzaki H, Matsubara M, Fujita K, Matsumura S, Suzuki S. Treatment of gastric remnant cancer post distal gastrectomy by endoscopic submucosal dissection using an insulation-tipped diathermic knife. *World J Gastroenterol* 2008; 14(16): 2550-2555 Available from: URL: <http://www.wjgnet.com/1007-9327/14/2550.asp> DOI: <http://dx.doi.org/10.3748/wjg.14.2550>

INTRODUCTION

Distal gastrectomy has been widely accepted as a standard operation for early stage gastric cancer. Gastric cancer of the proximal gastric remnant is now increasing in Japan. Nozaki *et al*^[1] described that the gastric remnants of 457 cases post distal gastrectomy due to gastric cancers were surveyed periodically by endoscopic examination, and 10 patients (2.2%) were diagnosed as having gastric remnant cancer within 12 years. Endoscopic submucosal dissection (ESD) techniques using a variety of knives, such as the insulation-tipped diathermic knife (IT-knife), hook knife, needle knife, or flex knife, have been developed in Japan^[2-10] and high one-piece resection rates have been reported for the treatment of mucosal malignancies^[3,11-17]. The remnant stomach post distal gastrectomy has narrow spaces. Thus, in this condition, it is more difficult to completely resect gastric remnant cancer by endoscopic technique than from an unoperated stomach. Few reports have described the results of ESD using an insulation-tipped diathermic knife (IT-ESD) for gastric remnant cancer. The purpose of this study was to evaluate the effectiveness of IT-ESD for patients with gastric remnant cancer post distal gastrectomy.

MATERIALS AND METHODS

Patients

We retrospectively reviewed the records of post distal gastrectomy patients with gastric remnant cancer, who underwent distal gastrectomy for the treatment of gastric cancer, admitted to Sumitomo Besshi Hospital and Shikoku Cancer Center between January 1998 and December 2007. Patients admitted during this period were divided into a conventional endoscopic mucosal resection (EMR) group and into a IT-ESD group according to the endoscopic resection method. Since 2000, the IT-knife procedure has been used instead of the conventional EMR for the treatment of gastric remnant cancer. Patient backgrounds, the one-piece resection rate, complete resection (CR) rate, operation times, bleeding rate, and perforation rate were compared between the groups. Patients with severe underlying disease, such as heart disease, respiratory disease, liver disease, or bleeding tendency, were excluded from the indication of ESD in our institute; however, no patients had severe underlying disease in this study. Patients taking drugs to promote bleeding, such as ticlopidine, aspirin, or warfarin, underwent ESD after a fixed term of drug discontinuance. All patients fulfilled the following criteria in this study: diagnosed as having mucosal gastric carcinoma by endoscopic findings or endoscopic ultrasonography, a biopsy specimen obtained from the lesion revealed differentiated adenocarcinoma, without ulceration of the lesion, no residual/local recurrence lesion after endoscopic treatment and the diameter of the lesion was up to 30 mm. The performance status (PS) of each patient was less than 2 on the Eastern Cooperative Oncology Group (ECOG) scale. IT-ESD was performed under informed consent. Two highly skilled endoscopists familiar with EMR and ESD performed endoscopic resection in this study.

EMR technique

Conventional EMR was performed with a 2-channel endoscope^[16,18]. The mucosa around the lesion was marked with coagulation current, and saline containing 0.0025% epinephrine was injected until the mucosa around or under the lesion was raised. Grasping forceps was passed through the loop of the conventional polypectomy snare, and an area near the lesion was grasped with the forceps to elevate the lesion. Then the snare was opened and the lesion was strangled. The specimen was completely resected by electric current application.

IT-ESD technique

IT-knife was developed by Hosokawa and Yoshida in 1994 in Japan^[19]. IT-knife has a ceramic ball at the top of the incising needle knife^[11,12,19-21] to prevent perforation of the gastric wall. IT-ESD was performed as previously described using a single-channel endoscope^[3,12,14,17,22]. Marks were made at several points along the outline of the lesion with a coagulation current, and saline containing 0.0025% epinephrine was injected just outside the marks to prevent perforation until the mucosa around the lesion was raised. A circumferential incision in the mucosa just outside the marks was made using an IT-knife, with a safe lateral margin, and the submucosal tissue under the circumcised

area was abraded with it. The specimen was then either completely resected using the IT-knife, or finally removed using a conventional polypectomy snare if it was attached only to a pedicle (Figure 1).

Histological assessment

A gastrointestinal pathologist evaluated the ESD specimens with special attention to the depth of tumor invasion, and the lateral and deep margins of the excision. Resected specimens were cut into 2 mm slices according to the Japanese Classification of Gastric Carcinoma^[23] and evaluated as to whether cancerous glands were present or absent at the margin of each slice.

Definition of complete and incomplete resection

When one-piece resection could be performed, it was easy to evaluate the completeness of the resection histologically. The efficacy of resection was determined according to the completeness of the resection: when the tumor was resected as a single piece, contained within the mucosal layer, and when the margin was definitely free of tumorous glands, resection was considered to be complete. Multifragment resections were defined as incomplete when tumorous glands were present in 2 or more fragments histologically, even if endoscopically the lesion had been completely removed. If the lateral margin of the lesion could not be evaluated histologically because of the effects of the electrosurgical current or mechanical damage, the resection was defined as being incomplete.

Complications

Bleeding and perforation were two major complications of ESD. Bleeding (delayed bleeding) was defined as clinical evidence of bleeding as hematemesis or melena at 1 d-10 d after ESD and requiring endoscopic treatment.

Statistical analysis

Values are expressed as the mean \pm SD. Statistical analysis was performed using the unpaired Student's *t*-test and the chi-square test. A *P* value of < 0.05 was considered significant.

RESULTS

Clinicopathological features of the tumors

Concerning tumor location and gross type, no significant differences were found between patients included in the EMR ($n = 15$) and those included in the IT-ESD ($n = 17$) group (Tables 1 and 2, and Figure 2).

Patient backgrounds

The mean age was 68.3 ± 9.2 years (range, 46-80 years) in the EMR and 73.1 ± 5.4 years (range, 64-84 years) in the IT-ESD group. The mean size of the lesion was 12.7 ± 2.9 mm and 15.5 ± 5.6 mm in the EMR and the IT-ESD group, respectively, with no significant difference between the two groups. No significant differences were found in the rate of underlying cardiopulmonary disease between the two groups [2 patients (13%) in the EMR group and 2 patients (12%) in the IT-ESD group] nor in

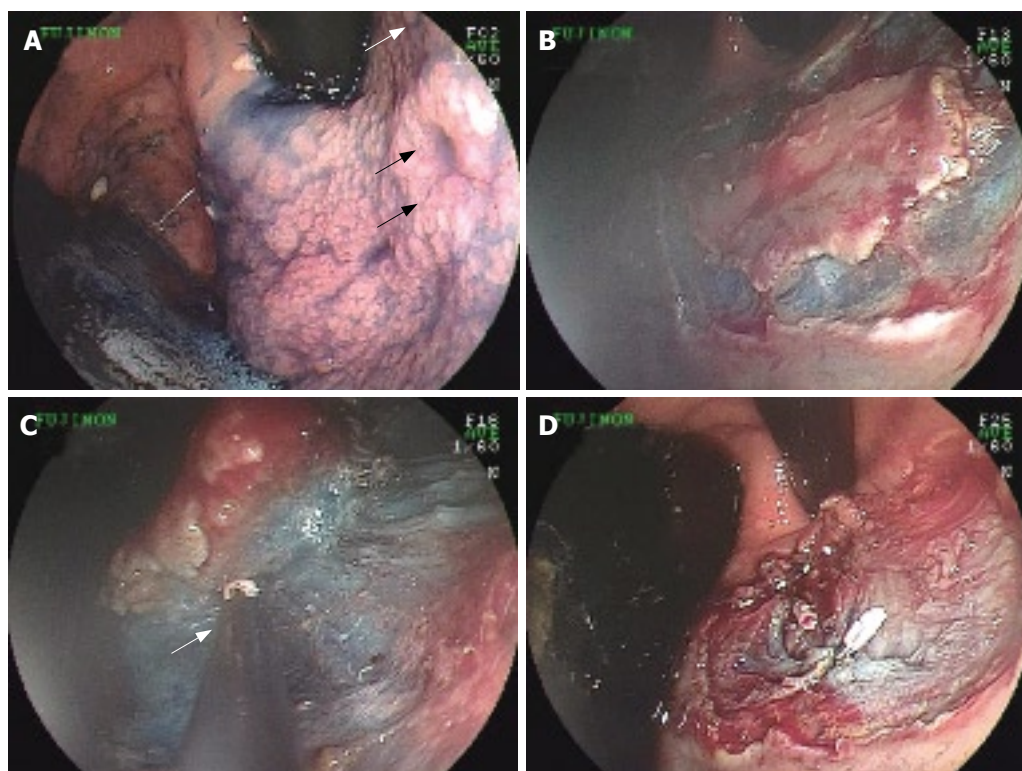


Figure 1 The procedure of endoscopic submucosal dissection using an insulation-tipped diathermic knife (IT-ESD). **A:** A II c lesion (black arrows) is seen near the suture line (white arrow) in the remnant stomach; **B:** Completion of IT-knife cutting around the lesion with a safe lateral margin; **C:** Abrasion of the submucosal tissue under the circumscribed area with IT-knife (white arrow); **D:** the tumor is completely resected with IT-knife.

Table 1 Tumor characteristics of patients in the ESD and EMR groups

	ESD group	EMR group
<i>n</i>	17	15
Location of the tumor (%)		
Posterior wall	4 (23.5)	3 (20)
Anterior wall	4 (23.5)	2 (13)
Lesser curvature	6 (35)	6 (40)
Greater curvature	3 (18)	4 (27)
Gross type of the tumor (%)		
Superficial depressed type	11 (65)	10 (67)
Superficial elevated type	6 (35)	5 (33)

the number of patients receiving anticoagulant therapy [one patient (6.7%) in the EMR group *vs* one patient (5.9%) in the IT-ESD group].

Evaluation of resected specimens and clinical outcomes

There was no significant difference in the one-piece resection rate among the groups (73% in the EMR group *vs* 100% in the IT-ESD group). The CR rate was significantly higher in the ESD group (40% in the EMR group *vs* 82% in the IT-ESD group). The mean operation time was 21.1 min \pm 12.2 min for the EMR group and 57.6 min \pm 31.9 min for the IT-ESD group. There were 4 cases of bleeding after endoscopic resection: one (6.7%) in the EMR group and 3 (18%) in the IT-ESD group, but no patient needed further surgery. All 3 patients with delayed bleeding developed clinical signs of hematemesis or melena 1 or 2 d after ESD. No patient developed hematemesis or melena later than 3 d after ESD. There was no severe delayed bleeding with a need for transfusion. Perforation

Table 2 Characteristics and results of clinical data of patients in the ESD and EMR groups

	ESD group (tumor size: 10-26 mm)	EMR group (tumor size: 10-18 mm)	<i>P</i>
<i>n</i>	17	15	
Mean age (yr)	73.1 \pm 5.4	68.3 \pm 9.2	NS
Mean size of the lesion (mm)	15.5 \pm 5.6	12.7 \pm 2.9	NS
Frequency of the cardiopulmonary underlying disease (%)	12	13	NS
Frequency of the anticoagulant therapy (%)	5.9	6.7	NS
One piece resection rate (%)	100	73	NS
Complete resection rate (%)	82	40	< 0.05
Operation time (min)	57.6 \pm 31.9	21.1 \pm 12.2	< 0.001
Complications			
Rate of bleeding (%)	18	6.7	NS
Rate of perforation (%)	0	0	NS
Depth of invasion (mucosa:submucosa)	15:2	14:1	

NS: Not significant.

occurred in none of the patients. Twelve patients were judged as having incomplete resection in both groups. The margin of the fragment was positive for tumor or the lateral margin of the lesion could not be evaluated because of the effects of the electrosurgical current in 5 patients (four in the EMR group and one in the IT-ESD group). These five patients did not undergo additional laser or surgical therapy, although they have remained under close periodic observation. Three patients (one in the EMR group and two in the IT-ESD group) were judged as having submucosal invasion and two underwent total gastrectomy

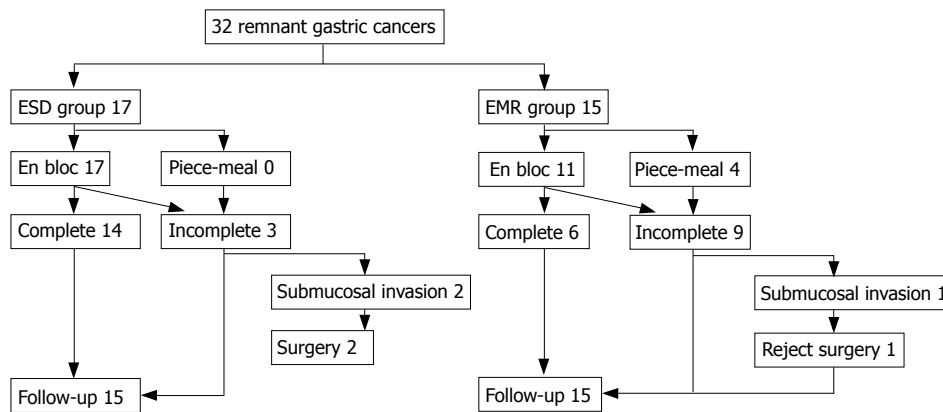


Figure 2 Clinical courses after endoscopic submucosal dissection (ESD) and conventional endoscopic mucosal resection (EMR) for remnant gastric cancers.

of the remnant stomach with regional lymph nodes dissection (additional surgery). The remaining case (in the EMR group) refused surgery and has been under close periodic observation. A total of 30 patients who did not undergo additional surgery were followed by endoscopy in both groups at least every year after treatment. The mean follow-up period was 2135 d (range 180–3285 d). There were no local recurrences after CR in the 20 patients in both groups and one patient resulting in incomplete resection in the IT-ESD group during that period. Only one patient in the EMR group resulted in piece-meal resection had a local recurrence 2 years after EMR and underwent additional EMR therapy.

DISCUSSION

EMR has become a standard treatment for intramucosal gastric cancer because it is less invasive for patients compared with surgical resection^[24–28]. To achieve cure by EMR, one-piece resection is optimal for all lesions because it may reduce the local recurrence rate^[10,29]. IT-ESD is a useful new endoscopic mucosal resection (EMR) method, which has recently become widespread in Japan due to its high one-piece resection rates^[3,11,13,15–17]. Few reports have described the results of IT-ESD for gastric remnant cancers in comparison with the usual strip biopsy method (conventional EMR).

Although PS for all patients was less than 2, and no patients had severe underlying diseases or severe complications in both groups, the CR rate was higher and operation time was longer in the IT-ESD group. In the present study, the one-piece resection rate and CR rate were high for over 10 mm lesions in the IT-ESD group. In contrast, the CR rate was low in the EMR group. In the unoperated stomach, it is easier to remove a tumor larger than 10 mm in diameter with IT-ESD than with the usual strip biopsy method^[11,22]. The reason why the one-piece resection rate and CR rate were high in the IT-ESD group in our study might be as follows: we adequately abraded the submucosal tissue under the lesion before snaring, and in more than half of the patients in this study, direct dissection of the submucosal layer was carried out with an IT-knife until complete removal had been achieved^[14,17]. The reason why the CR rate was low in the EMR group (under the condition of a remnant stomach) may be owing to the

difficulty of handling a polypectomy snare in the narrow space of the remnant stomach. In the IT-ESD method, the narrow space of the remnant stomach also makes IT-ESD procedures difficult; however, if the surgeon cuts around the lesion with a safe lateral margin, frequent CR is expected with the IT-ESD method. We speculated that it was difficult to ensure a safe lateral margin by snaring in the EMR technique because there were more patients with positive lateral margin in the EMR group (4/15) than in the IT-ESD group. Our study proved that the CR rate was significantly higher in the IT-ESD group, and IT-ESD was useful for gastric remnant cancers post distal gastrectomy that were larger than 10 mm in diameter, although it took longer.

The circulatory and respiratory states did not worsen in any patient during IT-ESD even if with underlying diseases or a long operation time. We should pay particular attention to patients with hypertension or anticoagulation therapy before or during ESD because they are related to bleeding during IT-ESD and the operation time^[14]. The good result in the IT-ESD group in this study might be due to the small number of patients having anticoagulation therapy. The operation time for IT-ESD was significantly longer than that of conventional EMR. The reasons why IT-ESD took longer than conventional EMR in this study might be as follows: complicated operation of the IT-knife for abrasion of the submucosal tissue, a high frequency of bleeding, and narrow space of the remnant stomach.

As for complications, the delayed bleeding frequency was 18% (3/17) in the IT-ESD group, which was higher than in Onozato *et al.*^[30] who reported it as 7.6%, although the total number of patients enrolled in this study was small. However, there were no problems due to delayed bleeding in this study in the IT-ESD group because we prevented delayed bleeding by follow-up endoscopic examination according to the clinical pathway^[3]. It is necessary to pay particular attention to perforation with the ESD technique^[13] because Ohkuwa *et al.*^[11], Miyazaki *et al.*^[13], Ono *et al.*^[20], and Fujishiro *et al.*^[31] reported that the incidence of perforation with IT-ESD is 5%–11.5%. We think that the risk of perforation with the ESD technique on a remnant stomach is higher than with the usual strip biopsy method on an unoperated stomach.

In conclusion, the CR rate was significantly higher in the IT-ESD group than in the EMR group, although the

operation time was significantly longer in the IT-ESD group. Our study proved that IT-ESD was an effective treatment for gastric remnant cancer post distal gastrectomy, and is useful for histological confirmation of successful treatment, although the long-term outcome should be evaluated in the future.

COMMENTS

Background

Endoscopic submucosal dissection (ESD) is a useful new endoscopic mucosal resection (EMR) method, which has recently become widespread in Japan due to its high one-piece resection rates. Gastric cancer of the proximal gastric remnant is now increasing in Japan. The aim of this study was to evaluate the effectiveness of ESD using an insulation-tipped diathermic knife (IT-ESD) for the treatment of patients with gastric remnant cancer post distal gastrectomy.

Research frontiers

It is still uncertain whether conventional EMR or IT-ESD results in better clinical outcome in the treatment of gastric remnant cancer post distal gastrectomy. Further investigations on a larger scale to compare the efficacy and safety of conventional EMR vs IT-ESD should be conducted.

Innovations and breakthroughs

In this article we analyze if IT-ESD is superior to conventional EMR in the complete resection rate in 32 patients with gastric remnant cancer post distal gastrectomy.

Applications

This study serves as a reminder to readers of the management of mucosal remnant gastric cancer post distal gastrectomy with IT-ESD. Because of its high complete resection rate, IT-ESD may be worth trying as the first therapy for treating patients with mucosal remnant gastric cancer post distal gastrectomy.

Peer review

It is an interesting initial series comparing conventional EMR to IT-ESD in the treatment of gastric remnant cancer post distal gastrectomy. The study presents novel findings.

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RAPID COMMUNICATION

Effect of biliary obstruction and internal biliary drainage on hepatic cytochrome P450 isozymes in rats

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Abstract

AIM: To investigate the total cytochrome P450 (CYP) content, microsomal mixed-function oxidase (MFO) activity, and expression of mRNAs for various CYP isozymes in a simple rat model of reversible obstructive jaundice.

METHODS: Obstructive jaundice was created in male rats by causing bile duct obstruction with polyester tape. In another group of rats, bile duct obstruction was followed by internal biliary drainage after releasing the tape. The expression of various CYP isozyme mRNAs was semi-quantitatively assessed by competitive RT-PCR.

RESULTS: The total CYP content and microsomal MFO activity showed a significant decrease after biliary obstruction, but returned to respective control levels after biliary drainage. A marked reduction in the expression of CYP1A2, 2B1/2, 2C11, 2E1, 3A1, and 3A2 mRNA was detected during biliary obstruction, while expression increased significantly toward the control level after biliary drainage. Although expression of CYP4A1 mRNA showed no reduction during biliary obstruction, it still increased significantly after biliary drainage.

CONCLUSION: These results suggest that not only obstructive jaundice, but also the subsequent internal biliary drainage may affect regulatory medications of the synthesis of individual CYP isozymes differently.

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Key words: Biliary obstruction; Obstructive jaundice; Biliary drainage; Mixed-function oxidase; P450 isozymes

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INTRODUCTION

A large number of drugs are metabolized in the liver. The hepatic cytochrome P450-dependent microsomal mixed-function oxidase (MFO) system plays a major role in the metabolism of both exogenous and endogenous substrates. It is well known that some drug-metabolizing enzymes are inhibited in cholestatic human liver^[1] while the total cytochrome P450 (CYP) content and MFO activity are reduced after bile duct ligation (BDL) in rats^[2-4]. A possible explanation for these changes has been suggested to be bile acid-mediated destruction of CYP hemoproteins.

CYP has multiple isozymes with different substrate specificities and the broad range of substrates for the hepatic MFO system is based on the existence of these multiple isozymes. These isozymes have been shown to be the hemeprotein products of the CYP supergene family^[5].

Previous studies have verified different susceptibility of BDL among CYP isozymes: male sex-specific CYPs (CYPs 2C11 and 3A2) than sexually undifferentiated CYPs (CYPs 1A, 2A1, 2B, 2C6, and 2E1)^[6,7]. There are also different alterations of hepatic CYP isozyme expression in patients with cirrhosis depending on the presence or absence of cholestasis^[1]. In patients with obstructive jaundice, biliary drainage has proved effective for both the treatment of jaundice itself and the subsequent complications. Ameliorating effect of biliary drainage on the total CYP content and MFO activities has been observed in bile duct obstructed rats^[8,9]; however, effects of biliary drainage on individual CYP isozymes remain unknown. In this study, using a simple model of reversible obstructive jaundice, the total CYP content, MFO activity and levels of mRNAs for various CYP isozymes were compared before and after internal biliary drainage in rats with bile duct obstruction.

MATERIALS AND METHODS

Animals and reversible obstructive jaundice model

Male Wistar rats, weighing 200–240 g were fed standard laboratory chow and had free access to tap water. All animals were handled according to the local institutional guidelines for the care and use of laboratory animals. Animals were randomly assigned to undergo either bile duct obstruction alone for 4 d or 10 d (d 4-BO and d 10-BO, $n = 5$ each) or bile duct obstruction for 4 d followed by drainage for 6 d (BOD, $n = 5$). Another 10 rats underwent a sham operation and served as controls for the BO and BOD rats (d 4-sham, $n = 5$ and d 10-sham, $n = 5$). Each rat was anesthetized with a subcutaneous injection of pentobarbital sodium (50 mg/kg), and placed in the supine position on the operating table. Reversible bile duct obstruction was produced by a modification of the method described by Posner *et al.*^[10]. Using a sterile technique, a midline abdominal incision was made and the porta hepatis was isolated. In BO and BOD rats, a 2-mm wide polyester tape was placed posterior to the bile duct at the hepatic hilum. The two ends of the tape were brought out through bilateral incisions at sites 1 cm lateral to the xiphoid process. Then the abdomen was closed in two layers with 2-0 surgical silk. Proper tension was applied to the bile duct with the tape, and two ends of the tape were fixed to the skin with 4-0 silk sutures. In BOD rats, the tape was carefully removed on the 4th postoperative day, allowing free internal bile flow. In sham operated rats, the same operation was performed, but the tape was placed loosely in order to avoid pressure on the bile duct. A blood sample was collected from each rat and analyzed with standard laboratory techniques for serum alanine aminotransferase (ALT) and total bilirubin. After blood collection, the rats were sacrificed and their livers were immediately removed. Then the livers were frozen in liquid nitrogen and stored at -80°C until use.

Preparation of hepatic microsomes

Livers were homogenized in a 4-fold volume of 0.15 mol/L KCl solution containing 10 mmol/L EDTA. The homogenate was centrifuged at 10 000 g for 15 min. The supernatant was centrifuged at 105 000 g for 60 min, the pellet was suspended, and centrifugation was done again. These preparations were performed at $0-4^{\circ}\text{C}$. The resulting pellet was suspended in 20 mmol/L potassium phosphate buffer (pH 7.4) containing 15% glycerol and used as hepatic microsomes.

Determination of the total hepatic microsomal CYP content and MFO activity

The concentration of hepatic microsomal protein was determined according to the method of Lowry *et al.*^[11]. The total CYP content was determined according to the method of Omura and Sato^[12]. Three types of MFO activity (i.e., aniline 4-hydroxylation, aminophylline N-demethylation and 7-ethoxycoumarin O-demethylation) were determined according to the methods described by Imai *et al.*^[13], Nash^[14], and Ullrich and Weber^[15], respectively. Each type of MFO activity was assayed by using NADPH as the sole electron source.

Determination of CYP mRNA levels by competitive RT-PCR

Total RNA was extracted from frozen hepatic tissue according to the method of Chomczynski and Sacchi^[16]. The resulting RNA was reconstituted in diethylpyrocarbonate-treated water, quantitated by spectrophotometry at 260 nm, and adjusted to 500 ng/ μL . The levels of mRNA for CYP1A2, 2B1/2, 2C11, 2E1, 3A1, 3A2, and 4A1 were determined with the rat cytochrome P450 competitive reverse transcriptase (RT) polymerase chain reaction (PCR) Set (Takara, Kyoto, Japan) and an RNA LA PCRTM Kit (Takara, Kyoto, Japan). Reverse transcription was performed in a final volume of 100 μL containing RT-buffer, 500 ng of total liver RNA, 5 μL of various concentrations of RNA competitor (1×10^7 , 4×10^7 , 1.6×10^8 and 6.4×10^8 copies/ μL), 5 mol/L MgCl_2 , 1 mol/L of each dNTP, 1 U/ μL of RNase inhibitor, 0.125 pmol/ μL of oligo dT-adaptor primer, and 0.25 U/ μL of AMV reverse transcriptase. Samples were incubated at 30°C for 10 min, 55°C for 20 min, 95°C for 5 min, and 5°C for 5 min. PCR amplification was performed in a final volume of 50 μL , containing PCR buffer, 10 μL of RT products, 5 mol/L MgCl_2 , 0.025 U/ μL of Taq DNA polymerase, and 0.2 $\mu\text{mol/L}$ each of the sense and antisense PCR primers. The primers were designed to specifically amplify the mRNA for CYP1A2, CYP2B1/2, CYP2C11, CYP2E1, CYP3A1, CYP3A2, CYP4A1, and cyclophilin. Cyclophilin is a housekeeping gene to correct for differences in RNA amounts between samples. Samples were incubated at 94°C for 2 min, followed by 24 cycles consisting of denaturation at 94°C for 30 s, annealing at 56°C for 30 s, and an elongation at 72°C for 30 s. Incubation of the samples was performed in a Gene Amp PCR system 9600 (Perkin-Elmer, USA). RT-PCR products were electrophoresed in 2% agarose gel. Then the gels were stained with ethidium bromide, and RT-PCR signals were visualized with UV light. Quantitation was performed by comparing RT-PCR signals generated from each specific CYP mRNA with the RT-PCR signals generated from varying concentrations of each RNA competitor.

Statistical analyses

Statistical analysis was done with the Mann-Whitney U test, and $P < 0.05$ was defined as indicating a significant difference between groups.

RESULTS

Characteristics of reversible obstructive jaundice model

No significant differences of the serum ALT and bilirubin levels were observed between the controls, d 4-sham rats, and d 10-sham rats. BO rats became jaundiced 4 d after bile duct obstruction, and their serum ALT and total bilirubin levels were significantly elevated compared with those of d 4-sham rats. At 6 d after the subsequent internal biliary drainage (BOD rats), these values showed a significant decrease compared with those in d 10-BO rats and were not significantly different from those in d 10-sham rats (Table 1).

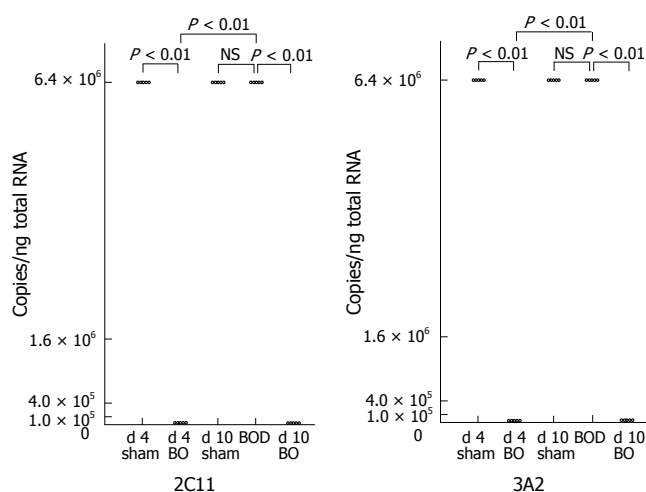
Total CYP content and MFO activity

The total CYP content and MFO activity showed

Table 1 Serum total bilirubin concentration, serum ALT concentration, the total cytochrome P450 content in hepatic microsomes and enzyme activities in hepatic microsomes from sham, bile duct obstruction alone and bile duct obstruction plus drainage groups (mean \pm SD, $n = 5$)

	d 4-sham	d 4-BO	d 10-sham	BOD	d 10-BO
Serum total bilirubin (mg/dL)	0.12 \pm 0.04	10.54 \pm 3.54 ^a	0.12 \pm 0.04	0.16 \pm 0.05 ^e	11.18 \pm 1.03
Serum ALT (U/L)	36.6 \pm 5.7	119.6 \pm 50.1 ^a	37.4 \pm 3.9	2.5 \pm 7.3 ^e	88.8 \pm 11.78
Total CYP content (nmol/mg MS protein)	0.577 \pm 0.029	0.378 \pm 0.012 ^a	0.668 \pm 0.080	0.603 \pm 0.080 ^e	0.329 \pm 0.05
Aniline 4-hydroxylation (nmol/mg MS protein/min)	0.527 \pm 0.054	0.261 \pm 0.026 ^a	0.568 \pm 0.068	0.570 \pm 0.091 ^e	0.252 \pm 0.03
Aminophylline N-demethylation (nmol/mg MS protein/min)	7.13 \pm 0.61	2.83 \pm 0.50 ^a	7.88 \pm 1.81	5.82 \pm 1.00 ^e	2.510 \pm 0.23
7-ethoxycoumarin O-demethylation (pmol/mg MS protein/min)	104.4 \pm 14.0	68.9 \pm 20.5 ^a	123.0 \pm 23.8	113.7 \pm 22.9 ^e	64.1 \pm 4.23

^a $P < 0.05$ vs d 4-sham; ^e $P < 0.05$ vs d 4-BO; ^e $P < 0.05$ vs d 4-BO and d 10-BO; d 10-BO and d 10-sham; BO: Bile duct obstruction alone; BOD: Bile duct obstruction plus drainage; CYP: Cytochrome P450.

**Figure 1** Effect of bile duct ligation and following internal biliary drainage on the mRNA expression of male-specific CYP isozymes. The mRNA expression was measured semi-quantitatively by competitive RT-PCR.

no significant differences between d 4-sham rats and d 10-sham rats. The total CYP content, aniline 4-hydroxylation activity, and 7-ethoxycoumarin O-demethylation activity were significantly decreased at 4 d after bile flow blockade and returned to the respective control levels after 6 d of internal biliary drainage. Aminophylline N-demethylation activity was also significantly decreased at 4 d after bile flow blockade and showed a significant increase after 6 d of internal biliary drainage, but did not return to the control level (Table 1).

CYP mRNA levels

CYP mRNA levels demonstrated no significant differences between d 4-sham rats and d 10-sham rats. The levels of CYP2C11, CYP3A2, CYP2B1/2, CYP2E1 and CYP3A1 mRNAs were significantly decreased at 4 d after bile flow blockade, and returned to the respective control levels after 6 d of internal biliary drainage (Figures 1 and 2). CYP1A2 mRNA also showed a significant decrease at 4 d after bile flow blockade and increased significantly after 6 d of internal biliary drainage, but did not return to the control level (Figure 3). CYP4A1 mRNA did not demonstrate significant change after 4 d of bile flow blockade, but showed a significant increase after 6 d of internal biliary drainage (Figure 3).

DISCUSSION

When patients suffer from obstructive jaundice caused by conditions such as choledocholithiasis or bile duct cancer, various drugs are administered to prevent subsequent complications (e.g. acute obstructive suppurative cholangitis or sepsis). Endoscopic or percutaneous transhepatic biliary drainage has also proved effective for the treatment of obstructive jaundice and its complications^[17-19]. Several studies have been conducted to ascertain the effects of bile duct obstruction on drug metabolism in the liver using animal^[2-4] models and human^[20], but the changes of hepatic drug metabolism following biliary drainage have not been clarified. Therefore, we investigated the total hepatic cytochrome P450 content, MFO activity, and expression of mRNA for various CYP isozymes prior to and following internal biliary drainage in rats with obstructive jaundice. The experimental model used in the present study was the internal biliary drainage model proposed by Posner *et al*^[10]. External biliary drainage models are unphysiologic because bile is excreted out of the body after the relief of obstructive jaundice. With other internal biliary drainage models such as choledochojunostomy, the effects of surgical invasion and anesthesia during internal biliary drainage must be taken into account. These events could affect CYP after following the relief of obstructive jaundice. On the other hand, the internal biliary drainage model used in the present study is more physiologic, and the impact of anesthesia and surgical invasion are relatively small. In rats with bile duct obstruction, irreversible fibrosis of the liver occurs after more than 14 d of bile duct obstruction^[21]. Therefore, in order to observe the changes of CYP during internal biliary drainage after acute obstructive jaundice, we performed internal biliary drainage after four days of bile duct obstruction.

In jaundiced patients, proinflammatory cytokines such as TNF or IL-6 are known to be released^[22], and an increase of TNF and IL-6 has been reported in murine obstructive jaundice models^[23]. On the other hand, it has been reported that both TNF and IL-6 decrease the expression of mRNA for CYP isozymes^[24-26]. These findings suggest that one of the mechanisms of reduced mRNA expression of CYP isozymes in obstructive jaundice may an increase in the level of cytokines such as TNF and IL-6.

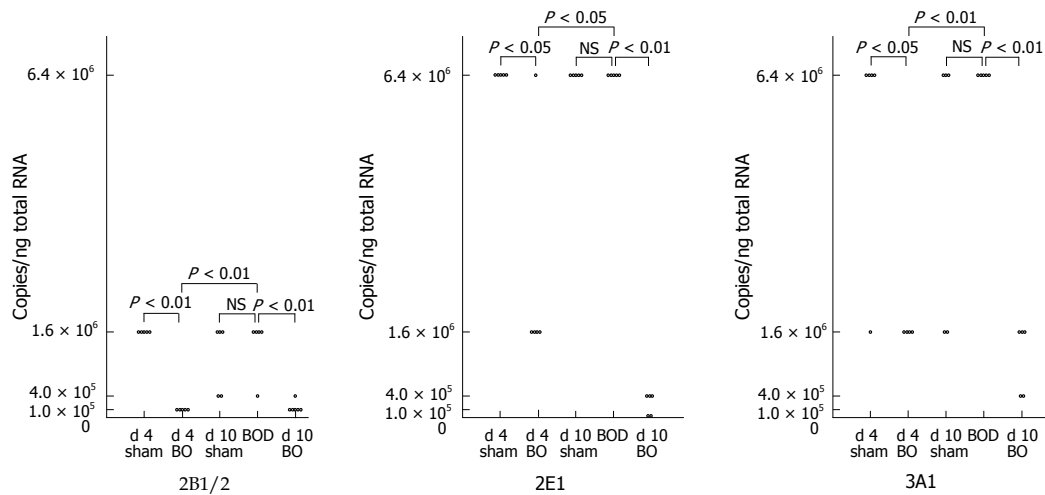


Figure 2 Effect of bile duct ligation and following internal biliary drainage on the mRNA expression of sexually undifferentiated CYP isozymes. The mRNA expression was measured semi-quantitatively by competitive RT-PCR.

The changes of the total CYP content and MFO activity following internal biliary drainage were comparable to those reported previously^[8]. In the present study, although the expression of mRNA for CYP isozymes decreased after bile duct obstruction, it returned to the control level after 6 d of internal biliary drainage, except for CYP1A2. The expression of CYP1A2 mRNA was also increased after 6 d of internal biliary drainage, but not to the control level. However, this does not exclude the possibility that the expression of CYP1A2 mRNA could return to the control level after more than 6 d of internal biliary drainage. The recovery of CYP mRNA expression can be explained as follows: factors that reduced expression of the various CYP isozymes after bile duct obstruction, such as changes of sex steroids and cytokines, were eliminated or reduced by internal biliary drainage. In addition, bile duct obstruction did not alter the expression of CYP4A1 mRNA, while internal biliary drainage increased its expression. Peroxisome proliferators such as clofibrate have been known to induce CYP4A1^[27,28]. It has been reported that LPS administration increases the expression of CYP4A1 mRNA in Fischer 344 rats^[29]. On the other hand, it has been reported that bile duct obstruction increases the serum estradiol concentration, and that estradiol suppresses the induction of CYP4A1 by clofibrate^[30]. The results of the present study showed that bile duct obstruction did not affect CYP4A1 mRNA expression although internal biliary drainage promoted of factors that increased its mRNA expression. However, it is also possible that bile duct obstruction produced multiple factors that both induced and suppressed CYP4A1 mRNA expression, so that there was no apparent change. Internal biliary drainage may then have selectively eliminated the suppressive factors and led to increased expression of CYP4A1 mRNA.

Clinically, patients with obstructive jaundice resulting from biliary disease such as choledocholithiasis or bile duct cancer are generally treated by biliary drainage, and the effectiveness of this treatment has been well documented^[17-19]. In the present study, bile duct obstruction increased the serum ALT and total bilirubin levels, but both returned to their control levels after 6 d of internal biliary drainage, and obstructive jaundice dissipated. Similarly, the reduced total CYP content returned to the control level. Nonetheless, the bile duct

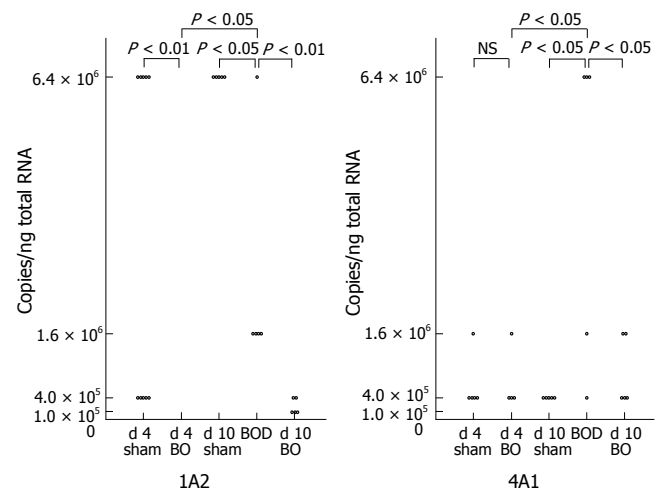


Figure 3 Effect of bile duct ligation and following internal biliary drainage on the mRNA expression of sexually undifferentiated CYP isozymes. The mRNA expression was measured semi-quantitatively by competitive RT-PCR.

obstruction and internal biliary drainage did not affect the expression of mRNA for the various CYP isozymes in the same manner. Internal biliary drainage did not result in sufficient recovery of the expression of mRNA for some CYP isozymes, and while bile duct obstruction had no effects, internal biliary drainage increased the expression of mRNA for some CYP isozymes. Therefore, even after the recovery of common indicators of obstructive jaundice, such as serum ATL and total bilirubin, hepatic drug metabolism could remain compromised. Caution should therefore be exercised when administering drugs following apparent recovery from obstructive jaundice.

Changes of CYP isozymes following obstructive jaundice and internal biliary drainage differed among the various isozymes, suggesting the existence of not simply a single mechanism, but multiple mechanisms. Further studies, such as the measurement of various cytokines, are therefore warranted in the future.

COMMENTS

Background

The hepatic cytochrome P450-dependent microsomal mixed-function oxidase

(MFO) system plays a major role in the metabolism of exogenous and endogenous substrates. Several authors have observed a decrease of the total hepatic cytochrome P450 (CYP) content and MFO activity in cholestatic bile duct ligation models. Since CYP consists of multiple isozymes with different substrate specificities, liver damage would probably affect various isozymes differently.

Research frontiers

This study focuses on the effect of biliary drainage on individual CYP isozymes in rats with bile duct obstruction. We used a model of reversible obstructive jaundice due to bile duct obstruction, and mRNA levels of different CYP isozymes were compared before and after internal biliary drainage.

Innovations and breakthroughs

There have been previous reports about the effects of biliary drainage on the total CYP content and MFO activity in rats with bile duct obstruction, but the effects of biliary drainage on individual CYP isozymes remain unknown. We demonstrated that bile duct obstruction and subsequent internal biliary drainage had various effects on the activity and mRNA expression of different CYP isozymes.

Applications

The effects of obstructive jaundice and internal biliary drainage differed among the various CYP isozymes. These results suggest the existence of multiple mechanisms that modify CYP activity, so further studies are warranted in the future.

Peer reviewer

This study used a simple model of reversible obstructive jaundice in rats to demonstrate that biliary obstruction and subsequent biliary drainage had a differential effect on the expression of mRNA for several CYP isozymes. The experiments were well designed and conducted.

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Effect of erythromycin on image quality and transit time of capsule endoscopy: A two-center study

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prolonged gastric emptying time.

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Abstract

AIM: To compare the effect of oral erythromycin *vs* no preparation with prokinetics on the transit time and the image quality of capsule endoscopy (CE) in evaluating small bowel (SB) pathology.

METHODS: We conducted a retrospective, blinded (to the type of preparation) review of 100 CE studies, 50 with no preparation with prokinetics from one medical center (Group A) and 50 from another center with administration of a single dose of 200 mg oral erythromycin 1 h prior to CE (Group B). Gastric, SB and total transit times were calculated, the presence of bile in the duodenum was scored, as was cleanliness within the proximal, middle and distal intestine.

RESULTS: The erythromycin group had a slightly shorter gastric transit time (21 min *vs* 28 min, with no statistical significance). SB transit time was similar for both groups (all $P > 0.05$). Total transit time was almost identical in both groups. The rate of incomplete examination was 16% for Group A and 10% for Group B ($P = 0.37$). Bile and cleanliness scores in different parts of the intestine were similar for the two groups ($P > 0.05$).

CONCLUSION: Preparation for capsule endoscopy with erythromycin does not affect SB or total transit time. It tends to reduce gastric transit time, but it does not increase the cecum-reaching rate. Erythromycin does not adversely affect image quality. We consider the routine use of oral erythromycin preparation as being unjustified, although it might be considered in patients with known

INTRODUCTION

Capsule endoscopy (CE) is a well accepted methodology which provides a direct and noninvasive way to view the entire small bowel (SB) mucosa^[1-6]. Capsule technology has enriched our knowledge about small bowel pathology and revolutionized the management of SB diseases^[7,8]. The best way to ensure the most complete and high-quality visualization of the entire small bowel is, however, a subject of controversy in the literature. In addition to improving the technical characteristics of the capsule itself, two ways of achieving these aims are proper preparation and the use of prokinetics^[9]. Decreasing gastric and SB transit times were expected to allow the capsule to successfully reach the cecum and to overcome the problem of the capsule's too-short battery lifetime (7 ± 1 h).

Erythromycin is a well-known prokinetic agent. It induces high amplitude gastric propulsive contractions by the initiation of gastric interdigestive migrating motor complexes. As a result, it accelerates gastric emptying, including that of indigestible particles^[10-12]. The effect of erythromycin on small bowel motility is less known. As such, its use to accelerate capsule transit time in the stomach and probably in the small intestine seemed to be an attractive idea, and several studies were conducted to explore this possibility^[13-15]. The number of such studies is very small and each includes few patients. Another aspect that has not yet been investigated is whether or not erythromycin stimulates the biliary-pancreatic secretions

to the small intestine, thereby adversely affecting image quality.

Uncertainty about these preparation-related issues of CE led to the uses of different CE preparation protocols among medical centers. Two Israeli medical centers have been routinely following two different protocols: patients undergoing CE at the Tel-Aviv Sourasky Medical Center (TASMC) are instructed to undergo a standard 12-h fast while those is examined at the Hillel-Yaffe Medical Center (HYMC) fast for 12 h and also take oral erythromycin to accelerate gastric emptying. The objective of the current study was to compare the CE data from these two medical centers in order to determine whether the addition of erythromycin leads to a relative acceleration of gastric and small intestinal transit times of the capsule without adversely affecting its image quality.

MATERIALS AND METHODS

This retrospective study was conducted in two Israeli medical centers, TASMC and HYMC. The protocol of the study was approved by the local Helsinki committees of both centers. The films of 50 capsules were randomly chosen from each center's Department of Gastroenterology's archives between 2000 and 2007. All 50 of the TASMC patients underwent a standard 12-h overnight fast preparation protocol (Group A), while all 50 of the HYMC patients followed a protocol that involved a 12-h overnight fast and a single dose of 200 mg oral erythromycin taken 1 h prior to undergoing the test (Group B). The CE procedure was standard in both centers: all patients ingested a PilliCam™ SB wireless video capsule (Given® Diagnostic Imaging System, Yokneam, Israel) with a small amount of water. An array of 8 sensors was attached to the abdominal wall, and a belt holding a recorder with a battery was fastened around their waists. Patients were allowed to drink 2 h after ingesting the capsule and to eat a light meal 4 h later. Eight h after capsule ingestion, the recorder was disconnected and the sensors were removed. The recorded digital information was downloaded onto the computer and the images were analyzed using RAPID® software. The provenance of the films was concealed from an independent expert who reviewed all 100 films.

The following data were collected from the CE studies and the CE endoscopy reports of all participating patients: demographics, the indications for referral to the procedure, the findings in the small intestine, and the rate of cecal arrival (i.e., the completeness of evaluation). The gastric transit time was calculated from the first view of the gastric mucosa to the first image of the duodenum and it was expressed in minutes. The SB transit time was calculated from the first view of the duodenum up to the first cecal image and it, too, was expressed in minutes. If the capsule did not reach the cecum during the battery's lifetime, the SB transit time was calculated as 480 min (8 h) minus gastric transit time.

The presence of bile in the duodenum lumen was evaluated using a scale of 1 to 4, with 1 representing none and 4 indicating more than 90% of the lumen being full of bile (Figure 1). The quality of visualization of SB mucosa

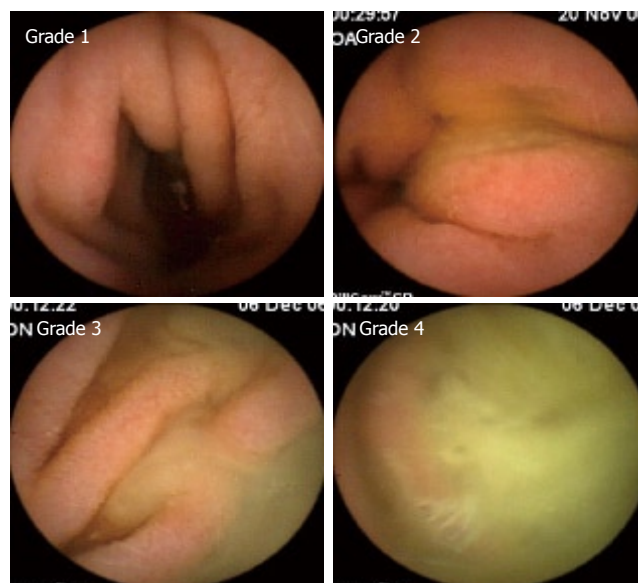


Figure 1 Grading of bile presence in the duodenum.

(i.e., cleanliness) was also evaluated by a 4-point scale, with 1 representing no residual material in the lumen and 4 indicating more than 90% of the lumen having residual material. The SB section of the CE study was divided into three parts, proximal, middle and distal intestine, and each was given a separate grading of cleanliness. A CE study was defined as having been completed if the capsule reached the cecum.

Comparisons between patients with and without erythromycin preparation with regard to demographic (age, gender) and clinical parameters (transit time, indications, diagnosis, *etc*) were performed using the Chi-square, Fisher's Exact and unpaired t-tests. The non-parametric Mann-Whitney test was applied since the continuous parameters did not follow a normal distribution. The relationships between transit time and other continuous parameters were examined by the Spearman's correlation coefficients. This was done for the entire sample and for each group separately (according to preparation type). The statistical analysis was performed by the SPSS for Windows software (Chicago, USA), version 14.0. The statistical tests were defined as having a confidence interval of 95%. A *P* value < 0.05 was considered significant for all tests performed.

RESULTS

A total of 100 CE studies were reviewed by an independent expert who was blinded to the center-belonging of the films. Fifty patients were from TASMC who used no preparation (Group A) and 50 patients were from HYMC who used an erythromycin preparation (Group B). In two studies (one from TASMC and one from HYMC), the gastric transit time was extremely prolonged (longer than 3 h) and, therefore, each of those studies was replaced by a randomly chosen one from the each center's archives.

Table 1 summarizes the study cohort's demographic and other relevant clinical data. Both groups were fairly similar, with the exception of the greater number of cases

Table 1 Demographic and clinical data of the study patients (*n* = 100)

Parameter	No erythromycin (<i>n</i> = 50)	Erythromycin (<i>n</i> = 50)	<i>P</i>
Age, yr (mean ± SD)	51.7 ± 21.6	52.1 ± 19.4	0.93 ¹
Male/Female	32/18	36/14	0.39
Indications			0.40
Suspected Crohn's	19	18	0.84
Obscure GI bleeding	23	28	0.32
Others ²	8	4	0.22
Final diagnosis in SB			0.10
Normal findings	23	19	0.42
Non-specific findings	9	7	0.59
Crohn's disease	1	7	0.06
Angiodysplasia	7	2	0.16
Polyps	1	3	0.62
Others ²	9	12	0.46

¹Unpaired *t*-test; ²Includes Indication (abdominal pain, celiac and polyposis syndrome) and final diagnosis (nodular hyperplasia, edematous fold, *etc*); the rest of *P* values are according to nonparametric Mann-Whitney test. SB: Small bowel; GI: Gastrointestinal.

Table 2 Transit times, bile and cleanliness scores in the two study groups (mean ± SD)

	No erythromycin (<i>n</i> = 50)	Erythromycin (<i>n</i> = 50)	<i>P</i>
Gastric transit time (min)	28.36 ± 23.56	21.08 ± 15.13	0.07 ¹ 0.16
SB transit time (min)	270.42 ± 108.13	279.14 ± 103.89	0.83
Total transit time (min)	299.26 ± 108.18	300.22 ± 103.25	0.97
Did not reach the cecum (%)	8 (16%)	5 (10%)	0.37
Bile presence score	1.75 ± 0.61	1.76 ± 0.58	0.99
Cleanliness score			
Proximal SB	1.78 ± 0.76	1.77 ± 0.74	1.00
Middle SB	2.22 ± 0.75	2.16 ± 0.77	0.73
Distal SB	2.79 ± 0.65	2.75 ± 0.74	0.73

¹Unpaired *t*-test; the rest of *P* values are according to nonparametric Mann-Whitney test. SB: Small bowel.

with a final diagnosis of Crohn's disease belonging to Group B (i.e., 1 case in Group A *vs* 7 cases in Group B, *P* = 0.06).

Table 2 compares the transit times, the rates at which the capsule reached the cecum, and the bile and cleanliness scores between the two groups. There was a trend towards a shorter gastric transit time for the patients in Group B (28 min in Group A *vs* 21 min in Group B, *P* = 0.07, unpaired *t*-test). The Mann-Whitney test (which is more accurate in groups with high variability) did not, however, confirm this finding (*P* = 0.16). Nevertheless, there was a higher variability of gastric transit times among the patients within Group A compared with the variability within Group B, which had more uniformity (*P* = 0.076 according to Levene's test for equality of variances).

There were no group differences in SB transit time (270 min in Group A *vs* 279 min in Group B) or in total transit time (299 min *vs* 300 min, respectively; Table 2). The capsule did not reach the cecum during the battery's lifetime in 8 Group A cases (16%) compared with 5 Group B cases (10%, *P* > 0.05). There was no case of capsule

Table 3 Gastric transit time with respect to cecum reachability

	No erythromycin (<i>n</i> = 50)	Erythromycin (<i>n</i> = 50)	<i>P</i> value
Gastric transit time in cases failing to reach cecum (min)	36.75 ± 33.93 (<i>n</i> = 8)	22.2 ± 14.45 (<i>n</i> = 5)	0.52
Gastric transit time in cases where cecum was reached (min)	26.76 ± 21.22 (<i>n</i> = 42)	20.96 ± 15.36 (<i>n</i> = 45)	0.24
<i>P</i>	0.52	0.81	

Table 4 Transit times according to indication for capsule endoscopy in Crohn's disease and obscure gastrointestinal bleeding (OGIB)

Transit time	Indication	No erythromycin (<i>n</i> = 50)	Erythromycin (<i>n</i> = 50)	<i>P</i>
Gastric transit time (min)	Crohn's disease	27.16 ± 26.11	16.72 ± 13.21	0.19
	OGIB	29.82 ± 23.53	23.61 ± 16.49	0.43
	<i>P</i>	0.41	0.16	
Small bowel transit time (min)	Crohn's disease	285.53 ± 96.31	324.50 ± 114.57	0.28
	OGIB	230.39 ± 103.00	255.32 ± 90.81	0.44
	<i>P</i>	0.066	0.04	

retention. Even after excluding from the calculations those cases in which the capsule did not reach the cecum, there were still no significant differences between Group A and Group B in SB and total transit times: the respective SB transit times were 237.50 ± 82.69 min and 259.29 ± 89.28 min (*P* = 0.44), and the respective total transit times were 264.83 ± 79.99 min and 280.24 ± 88.27 min (*P* = 0.56).

A comparison of the presence of bile (a score of 1.75 in Group A *vs* 1.76 in Group B, *P* = 0.99) and of the cleanliness scores in different parts of the SB failed to show any differences as well (Table 2). As expected in each group, the presence of residual material was more prominent in the distal than in the proximal intestine, assuring a better visualization of the SB mucosa in the proximal intestine.

We sought to determine whether or not the gastric transit time in cases in which the capsule failed to reach the cecum was longer than in cases with completed tests, assuming that extended transit time may consume valuable battery time for gastric visualization than for the small intestine. It emerged that the gastric transit time was fairly similar for all subgroups, regardless of cecum reachability (Table 3).

An additional analysis was performed to explore the relationships between the patient's clinical picture and capsule transit times (Table 4). The two major indications for undergoing CE that were chosen for this assessment were a clinical picture suspicious for Crohn's disease (abdominal pain, diarrhea, elevated CRP, anemia, *etc*) and for obscure gastrointestinal (GI) bleeding (OGIB). There were no differences in gastric transit times between these two indications for either Group A or Group B or between the two groups. SB transit time was, however, significantly longer in the cases suspected to have Crohn's disease

than OGIB in Group B and almost significantly longer in Group A ($P = 0.04$ and $P = 0.063$, respectively). Neither the preparation with erythromycin nor the indication for CE affected the cecum-reaching rates in both groups ($P = 0.160$ and $P = 0.452$, respectively).

Age below or above 40 years old (decided arbitrarily) did not affect gastric or SB transit time, but there was some tendency for shorter total transit time in patients above age 40 only in Group A ($P = 0.07$). In addition, Group A demonstrated a borderline gender-dependent difference: females in Group A tended to have a shorter gastric transit time and a longer SB transit time ($P = 0.052$ and $P = 0.069$, respectively). No influence of age or gender on transit times was evidenced in Group B.

DISCUSSION

The results of our comparative two-center study demonstrate that preparation with oral erythromycin before a CE study does not affect SB transit time, total transit time or the rate of the capsule in reaching the cecum. The medication may have had a possible shortening effect on gastric transit time and it tended to reduce the variability of gastric transit times among the patients in the group that used it. The length of gastric time does not, however, predict the likelihood that the capsule will reach the cecum during the battery's lifetime. We demonstrated that the indication for patients undergoing CE affects the SB transit time, but not any of the other examined parameters, and that the influences of age and gender are only marginal. Erythromycin does not affect adversely an image quality as measured by presence of bile and residual material in the intestine.

Our study is the largest thus far to address the issue of erythromycin preparation before a CE study. Leung *et al*^[14] conducted a small prospective comparative study on 24 patients who received an oral erythromycin preparation *vs* 14 patients who were not given any prokinetics. While no differences were found in SB transit times, in cecum arrival rates or in the quality of images, there was a highly significant difference in gastric transit time between these two groups (16 min *vs* 70 min, respectively, $P = 0.005$). Caddy *et al*^[13] performed a study of similar design (22 and 23 patients in each group) and demonstrated no significant difference in gastric and SB transit times, cecum arrival rate or image quality between the two groups. The third published study on this issue was conducted by Fireman *et al*^[15]. Their investigation included one group prepared with polyethylene glycol (26 patients), another with erythromycin (29 patients) and a third with no preparation (40 patients). According to their results, erythromycin had no effect on SB transit time, but it did show some tendency for shortening gastric transit time. The erythromycin group in Fireman *et al* study^[15] had significantly more residual material in the lumen and poorer image quality.

Altogether, there is general agreement among the available studies that erythromycin does not affect SB transit time but that it may or may not shorten gastric time. There are some minor differences in design between those earlier studies and our current one. We excluded the patients with extremely prolonged gastric emptying time (> 3 h) in order

to prevent skewing of the data during the statistical analysis (1 patient from Group A and 1 patient from Group B). In addition, the dosage of erythromycin used in the other studies was slightly higher than in ours (250 mg *vs* 200 mg, respectively) although the route of administration (oral) and timing (1 h prior to CE study) were identical. Our study was retrospective, but all the CE studies were re-examined by an independent expert who was blind to the hospital in which any given CE study had been performed.

The question about whether or not erythromycin stimulates the secretion of bile and by doing so impairs the image quality in the duodenum had not been previously explored in CE studies. Using a bile score, we could now clearly show that there is no such stimulation of bile secretion. As for the controversial issue of erythromycin's affecting the cleanliness score, we used a separate score for the proximal, middle and distal parts of the intestine and found no negative impact of the medication on the cleanliness of the intestine.

In conclusion, we suggest that the routine use of oral erythromycin 200-250 mg for SB or total transit time in CE is unfounded. It should, however, be considered as part of the preparation for patients with known prolonged gastric emptying time (i.e. either from a previous CE study or other imaging tests) or in patients with symptoms characteristic of gastroparesis (e.g. feeling of upper abdominal fullness, vomiting, *etc*). The optimal dosage (probably higher dosage) and the preferred route of administration of erythromycin (intravenous *vs* oral) in the setting of CE await further study.

ACKNOWLEDGMENTS

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COMMENTS

Background

Capsule endoscopy (CE) is a well accepted methodology which provides a direct and noninvasive way to view the entire small bowel (SB) mucosa. The best way to ensure the most complete and high-quality visualization of the entire small bowel is, however, a subject of controversy in the literature. The aim of this study is to compare the effect of oral erythromycin *vs* no preparation with prokinetics on the transit time and the image quality of CE in evaluating SB pathology.

Research frontiers

We conducted a retrospective, blinded (to the type of preparation) review of 100 CE studies, 50 with no preparation with prokinetics from one medical center (Group A) and 50 from another center with administration of a single dose of 200 mg oral erythromycin 1 h prior to CE (Group B). The results of the study demonstrated that preparation for capsule endoscopy with erythromycin does not affect SB or total transit time ($P > 0.05$). It tends to reduce gastric transit time ($P = 0.07$), but it does not increase the cecum-reaching rate ($P = 0.37$). Erythromycin does not adversely affect image quality (both bile score and the score of cleanliness). We consider the routine use of oral erythromycin preparation as being unjustified, although it might be considered in patients with known prolonged gastric emptying time.

Innovations and breakthroughs

Our study is the largest thus far to address the issue of erythromycin preparation before a CE study. In addition, we used a new bile score and a separate score of cleanliness for different parts of the small intestine. These innovations made the conclusions more precise. Our study was retrospective, but all the CE studies were re-examined by an independent expert who was blind to the hospital in which

any given CE study had been performed. We concluded that the routine use of oral erythromycin 200 mg-250 mg for SB or total transit time in CE is unfounded. It should, however, be considered as part of the preparation for patients with known prolonged gastric emptying time (i.e. either from a previous CE study or other imaging tests) or in patients with symptoms characteristic of gastroparesis (e.g. feeling of upper abdominal fullness, vomiting, *etc*).

Applications

The conclusions of our study may spare to the patients an unnecessary use of antibiotics before the capsule procedure.

Peer review

This is a retrospective comparative study comparing two preparation regimens for capsule endoscopy. One group received oral erythromycin and the second did not receive a prokinetic preparation. The study aimed to look for differences in capsule transit time, but was not able to demonstrate an advantage for the erythromycin group. The paper is well written and the study design although retrospective adequate.

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RAPID COMMUNICATION

Hepatoprotective activity of *Sapindus mukorossi* and *Rheum emodi* extracts: *In vitro* and *in vivo* studies

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on primary hepatocytes cultures and in *in vivo* in a rat model of CCl₄ mediated liver injury.

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Abstract

AIM: To study the hepatoprotective capacity of *Sapindus mukorossi* (*S. mukorossi*) and *Rheum emodi* (*R. emodi*) extracts in CCl₄ treated male rats.

METHODS: The dried powder of *S. mukorossi* and *R. emodi* was extracted successively with petroleum ether, benzene, chloroform, and ethanol and concentrated in vacuum. Primary rat hepatocyte monolayer cultures were used for *in vitro* studies. *In vivo*, the hepatoprotective capacity of the extract of the fruit pericarp of *S. mukorossi* and the rhizomes of *R. emodi* was analyzed in liver injured CCl₄-treated male rats.

RESULTS: *In vitro*: primary hepatocytes monolayer cultures were treated with CCl₄ and extracts of *S. mukorossi* & *R. emodi*. A protective activity could be demonstrated in the CCl₄ damaged primary monolayer culture. *In vivo*: extracts of the fruit pericarp of *S. mukorossi* (2.5 mg/mL) and rhizomes of *R. emodi* (3.0 mg/mL) were found to have protective properties in rats with CCl₄ induced liver damage as judged from serum marker enzyme activities.

CONCLUSION: The extracts of *S. mukorossi* and *R. emodi* do have a protective capacity both *in vitro*

INTRODUCTION

The liver is an organ of paramount importance. Due to its unique and considerable regenerative capacity, even a moderate cell injury is not reflected by measurable change in its metabolic functions. However, some of its functions are so sensitive that abnormalities start appearing depending upon the nature and the degree of initial damage.

The etiology of the liver disorders depends on various factors as nutritional, biochemical, bacteriological, viral, or environmental aberration. The liver plays a significant role not only in the metabolism and disposition of the chemicals to which it is exposed directly or indirectly, but also in the metabolism of fats, carbohydrates, proteins, and immunomodulation.

The impairment of the liver function is generally caused by xenobiotics, excessive exposure to various pharmacological and chemical agents, and protozoal or viral infections. Depending upon the severity of cellular injury, acute hepatitis can lead to chronic hepatitis, which is finally terminated to cirrhosis or malignant lesions in untreated cases. In case of deranged liver functions, the chemical composition of liver or its subcellular organelles are possibly altered. A slight alteration in hepatic structure and function may result in portal hypertension, ascities, jaundice, and increased bleeding, and causes multiple metabolic changes affecting other organs as well. Medical

survey indicates exceptionally high occurrence of hepatic diseases and has become one of the most serious problem in the area of public health. Acute viral hepatitis is a diffuse inflammatory lesion of the liver usually accompanied by clinical and biochemical abnormalities and often caused by the well-characterized hepatitis virus. Acute hepatitis closely resembles viral hepatitis clinically, biochemically, and histologically. It can also be induced by chemicals or drugs.

Alcoholic liver disease (ALD) is one of the most serious consequences of chronic alcohol abuse. Liver cirrhosis, the culmination of the illness, is one of the leading causes of death in western countries^[1,2].

According to Wang^[3] chronic and excessive ethanol consumption is associated with cellular proliferation, fibrosis, cirrhosis, and cancer of the liver. An important characteristic of alcohol-induced liver injury is an impaired vitamin A nutritional status. Studies in human Hep G2 cells have shown that ethanol is cytotoxic to Hep G2 cells, which are transduced to express P-450 2E1 (CYP 2E1) and this toxicity is apoptotic in nature^[4] predominantly in the liver. The main pathways for hepatic oxidation of ethanol to acetaldehyde involve alcohol dehydrogenase^[5] and are associated with the reduction of NAD⁺ to NADH^[6].

The magnitude of derangement of liver by disease or hepatotoxins is generally measured by the level of glutamate pyruvate transaminase (ALT), glutamate oxaloacetate transaminase (AST), alkaline phosphatase (ALP), bilirubin, albumin, and whole liver homogenate.

Herbal drugs are playing an important role in health care programs worldwide, and there is a resurgence of interest in herbal medicines for treatment of various ailments including hepatopathy. India, the abode of Ayurvedic system of medicine, assigns much importance to the pharmacological aspects of many plants. Hepatoprotective effect of some plants like *Spirulina maxima*^[7], *Eclipta alba*^[8], *Boehmeria niveda*^[9], *Cichorium intybus*^[10], and *Picrorhiza kurroa*^[11] has been well established. Nearly 150 phytoconstituents from 101 plants have been claimed to possess liver protecting activity^[12]. At the same time, surprisingly, we do not have satisfactory plant drugs/formulations to treat severe liver diseases. Most of the studies on hepatoprotective plants are carried out using chemical induced liver damage in rodents as models. A few excellent reviews have appeared on this subject in the recent past^[13].

This study is based on the natural products responsible for repairing and healing of adversely affected liver cells. In the present study, we selected two plants namely *S. mukorossi* and *R. emodi* and investigated the hepatoprotective effect of these plant extracts against CCl₄ induced hepatocyte damage *in vitro* and liver injury *in vivo*.

S. mukorossi Gaerten (Sapindaceae), commonly known as Ritha or Aritha is found throughout India. The major constituents of its fruit are saponins (10%-11.5%), sugars (10%) and mucilage^[14]. The fruit of the plant is reported to have expectorant, emetic, alexipharmic, and abortifacient effects. It is also used in excessive salivation, epilepsy and chlorosis^[15,16]. Saponins from this plant are known to be spermicidal *in vitro*^[17]. This spermicidal property has been used in contraceptive cream^[18]. The alcoholic extract

(*Sapindus trifoliatum* Linn) is reported to possess anti-implantation activity.

R. emodi (Polygonaceae) commonly known as Indian or Himalayan Rhubarb is found in India. The major constituents of rhubarb rhizomes are anthraquinones. Rhubarb is used as a laxative, diuretic to treat kidney stones, gout, and liver diseases characterized by jaundice. Externally, it is used to heal skin sores and scabs. Paradoxically, although larger doses are used as laxative, small doses are used to treat dysenteric diarrhea^[19]. Chinese use rhubarb as an ulcer remedy and consider it a bitter, cold, dry herb used to "clear heat" from the liver, stomach and blood, to expel helminthes and to treat cancer, fever, upper intestinal bleeding (ulcers), and headache^[20,21]. It is also used to treat toothache^[22]. In Europe, rhubarb is a component of spring tonics or blood cleansing cures, including Swedish bitter^[23]. Turkish or medicinal rhubarb is also one of the four major ingredients in the herbal cancer remedy.

We isolated the extracts from both plants, and a study was designed using the products of *S. mukorossi* and *R. emodi* to assess the hepatoprotective effect of these plant extracts against CCl₄ induced hepatocyte damage *in vitro* and liver injury *in vivo*.

MATERIALS AND METHODS

Plant materials

Authentic samples of *S. mukorossi* and *R. emodi* were obtained from authorized supplier M/s Munnalal Dawasas and Co. Hyderabad, Andhra Pradesh, India. The plants were previously identified and authenticated by experts in the Post Graduate and Research Department of Botany, Anwar-ul-loom College Hyderabad, Andhra Pradesh, India.

Animals

Male Wister rats weighing 175-200 g were obtained from the animal house of Deccan College of Medical Sciences, Hyderabad and housed in polycarbonate cages. The rats had free access to standard pellet chow and water *ad libitum* throughout the experiment with the exception of some experiments (see below) in which the animals were deprived of food, but not water, for 18-24 h before the experiments were performed. After procurement, all the animals were divided into different groups and were left for one week for acclimatization to experimentation room and were maintained on standard conditions (23°C, 60%-70% relative humidity and 12 h photo period). There were six animals in each group for observational screening and acute toxicity studies. All experimental protocols described below were approved by the ethical board.

Extraction, separation, and purification of the compounds

For phytochemical analysis, approximately 100 g of fruit pericarp of *S. mukorossi* and rhizomes of *R. emodi* was collected and materials were chopped, air dried at 35-40°C and pulverized in electric grinder. The powder obtained was successively extracted with the following chemicals, petroleum ether (60-80)°C, benzene, chloroform, and ethanol, respectively.

The extracts were then powdered by using rotary evaporator under reduced pressure. Fruit pericarp of *S. mukorossi* yielded 38 g, 28 g, 34 g, and 35 g and rhizomes of *R. emodi* yielded 19 g, 17 g, 21 g, and 22 g powdered extracts with petroleum ether, benzene, chloroform, and ethanol, respectively.

The extracts were obtained by percolation using 70% of ethanol as solvent at room temperature; according to process A of Farmacopeia dos Estados Unidos do Brasil (1959) (AOAC 1990). The extracts were evaporated at 40°C under vacuum and the residue was freeze-dried. The dry extracts of the fruit pericarp of *S. mukorossi* and rhizomes of *R. emodi* were tested for the presence of saponins and anthraquinones.

Each extract of the fruit pericarp of *S. mukorossi* (SM) and rhizomes of *R. emodi* (RE) were column chromatographed over Silica gel (200 mesh), eluting with CHCl₃-MeOH (70:30, 60:40, 50:50, 25:75) and compound fractions of (250 mL each) were collected and monitored by TLC. These column chromatographed compound fractions were further filtered to yield saponins from *S. mukorossi* and anthraquinones from *R. emodi*, which were separated by paper chromatography and preparative TLC to yield saponins [(SM-A (petroleum ether), SM-B (benzene), SM-C (chloroform) & SM-D (ethanol)], and anthraquinones [(RE-A (petroleum ether), RE-B (benzene), RE-C (chloroform) & RE-D (ethanol)], respectively. All the filtrates obtained were dried by evaporation (Rotometer, 40°C), the dried extracts were individually dissolved in 10 mL ethanol (95%) and then subjected to complete drying process and weighed according to the AOAC (1990) method^[20].

Hepatotoxins

It is emphasized that hepatotoxins that cause acute hepatitis should have close resemblance with the viral hepatitis, clinically, biochemically, and histologically. Certain drugs are also responsible for chronic hepatic disease such as chronic hepatitis, fatty liver, cirrhosis, and several vascular lesions of the liver. In many instances drug induced hepatitis is indistinguishable from viral hepatitis. Chemically induced hepatic injury for experimental studies should be severe enough to cause cell death or to modify hepatic functions. The mechanism of acute hepatic injury depends upon the chemical compound and the species of animals used. We have studied hepatoprotective activity against carbon tetrachloride (CCl₄) induced hepatotoxicity.

CCl₄ is one of the most powerful hepatotoxin in terms of severity of injury. It causes toxic necrosis leading to biochemical changes having clinical features similar to those of acute viral hepatitis^[24,25]. Liver injury was produced by administration of CCl₄ mixed with liquid paraffin. Animals were given single doses of CCl₄ 100 µL/kg *p.o.* per day through out the experimental setup. Control animals received an equal volume of liquid paraffin.

Methods for the hepatoprotective evaluation of extracts of *S. mukorossi* and *R. emodi*

In vitro: Fasting Wistar male adult rats weighing 280-300 g were used. Liver cells were isolated by using a modified procedure of Kiso *et al.*^[27]. The animal was cleaned

Table 1 *In vitro* cytotoxicity profile of extracts of *S. mukorossi* and *R. emodi* (mean ± SE)

Sample identity	Test concn (µg/mL)	Cytotoxicity (% release of enzyme in the medium)			
		LDH		GPT	
		Sp. activity	%leakage	Sp. activity	%leakage
Control	-	5.05 ± 0.22	-	103 ± 2.5	-
<i>S. mukorossi</i>	10	5.00 ± 2.21	NS	111 ± 2.4	NS
	50	4.97 ± 0.23	NS	108 ± 3.1	NS
	100	5.18 ± 0.31	NS	100 ± 1.9	NS
<i>R. emodi</i>	10	5.03 ± 0.11	NS	119 ± 2.0	NS
	50	5.12 ± 0.17	NS	107 ± 2.5	NS
	100	5.08 ± 0.14	NS	114 ± 2.3	NS

NS: Non-significant (t-test).

thoroughly using rectified alcohol and anaesthetized with ether. Dissection of the animal was carried out under aseptic conditions using sterilized instruments. A midline incision was made on the abdomen of the anaesthetized animal. The portal vein was cannulated with needle no 25 connected to an infusion set. The needle was tied in place and the inferior vena cava was cut below the renal vein. Perfusion of the liver was started immediately with Ca²⁺-Mg²⁺ free Hanks buffer salt solution (pH 7.4 at 37°C) which was prepared according to the procedure of Ohno (1965). When the liver was thoroughly perfused (*i.e.* has turned white), the flow of HBSS was stopped and the needle was removed. The liver was transferred to a sterile Petri dish containing Ca²⁺-Mg²⁺ free HBSS and minced into small pieces, which were transferred to a conical flask containing 10 mL of 0.075% collagenase in HBSS. This was placed on a magnetic stirrer at 37°C for 10 min. The cell suspension thus obtained was centrifuged at 50 g for 10 min. The supernatant was aspirated and the cell suspended in the Ca²⁺-Mg²⁺ free HBSS. The cells were washed twice and counted in the presence of trypan blue dye. Viability of the cells in each of the experiment performed was found to be 90%. The isolated hepatocytes were cultured in Eagles MEM, supplemented with 10% inactivated serum at density of 0.5 × 10⁹ cells/L in sterile disposable culture bottles and incubated in a humidified incubator at 37°C under 5% CO₂. The viability of hepatocytes was studied after 6, 12, and 24 hrs. The hepatocytes which settled down were observed for their growth. Cytotoxicity of *S. mukorossi* and *R. emodi* extracts was tested in primary hepatocytes monolayer cultures. Neither of the extracts caused significant enzymes release or were cytotoxic (Table 1).

Hepatocytes were prepared from male Wistar rats by the collagenase perfusion technique^[26]. Cells were purified by several centrifugations and inoculated at density of 0.5 × 10⁸ cells/L on collagen coated plates. One day after the isolated rat hepatocytes were plated, cells were exposed to medium containing 7 mmol/L CCl₄ with or without the sample to be tested for the hepatoprotective activity^[27]. After the exposure to CCl₄ for 1 h the culture medium was collected and used for the determination of different parameters.

Lipid peroxidation was assessed as TBA-reactive substances using malondialdehyde (MDA) as reference.

Table 2 *In vitro* hepatoprotective activity of *S. mukorossi* and *R. emodi* extracts (mean \pm SE)

Sample identity	Test concen (μ g/mL)	Hepatoprotective effect					
		GSH levels (cells)		[% restoration of enzyme leakage against toxin challenge (CCl ₄) of enzyme leakage]			
		μ g/3 \times 10 ⁶ cells	% recovery	LDH		GPT	
				Sp. activity	% restoration	Sp. activity	% restoration
Control	-	5.61 \pm 0.13	-	3.22 \pm 0.25	100	94 \pm 3.0	0
CCl ₄	2.5 mmol/L	2.52 \pm 0.14	-	16.0 \pm 0.22	0	198 \pm 2.5	0
CCl ₄ +	10 μ g	2.95 \pm 0.22	16	10.2 \pm 2.21 ^a	46	135 \pm 2.4 ^a	60
<i>S. mukorossi</i>	50 μ g	3.05 \pm 0.17	20 ^a	8.10 \pm 0.23 ^b	61	123 \pm 3.1 ^a	72
	100 μ g	3.31 \pm 0.19	34 ^a	6.80 \pm 0.31 ^b	71	105 \pm 1.9 ^b	89
CCl ₄ +	10 μ g	3.12 \pm 0.13	24 ^a	10.51 \pm 0.11 ^a	43	143 \pm 2.0 ^a	52
<i>R. emodi</i>	50 μ g	3.27 \pm 0.21	32 ^a	7.34 \pm 0.17 ^b	67	129 \pm 2.5 ^a	66
	100 μ g	3.30 \pm 0.13	33 ^a	6.08 \pm 0.14 ^b	77	108 \pm 2.3 ^a	86
CCl ₄ + Silymarin	30 μ g	4.61 \pm 0.22	69 ^a	6.00 \pm 0.50 ^b	78	107 \pm 2.3 ^a	87

^a*P* < 0.05, ^b*P* < 0.01 *vs* control.Table 3 Effect of *S. mukorossi* and *R. emodi* extracts on serum biochemical parameters against CCl₄ induced hepatic injury in rats (Curative study, mean \pm SE)

Treatment	Dose (mg/kg, <i>p.o.</i>)	Serum parameters			
		ALT (IU/L)	AST (IU/L)	ALP (IU/L)	Bilirubin (mg)
Vehicle control	-	127.73 \pm 10.65	100.26 \pm 11.50	40.11 \pm 2.20	0.11 \pm 0.02
Vehicle + CCl ₄	-	1142.36 \pm 80.97	833.59 \pm 34.85	64.06 \pm 3.00	0.82 \pm 0.08
<i>S. mukorossi</i> CCl ₄ +	2.5	524.18 \pm 86.30 ^b	408.72 \pm 40.07 ^a	47.38 \pm 1.02 ^b	0.40 \pm 0.02 ^b
<i>R. emodi</i> CCl ₄ +	3	384.69 \pm 44.39 ^b	314.90 \pm 44.41 ^b	49.00 \pm 2.19	0.32 \pm 0.02 ^b
Silymarin CCl ₄ +	50	563.47 \pm 49.22 ^b	421.54 \pm 30.82 ^b	47.96 \pm 2.49 ^b	0.42 \pm 0.02 ^b

^a*P* < 0.05, ^b*P* < 0.01 *vs* control.

Rat hepatocytes 1 \times 10⁹ cells/L were incubated in a final volume of 1.0 mL HBSS buffer containing test materials in presence of 200 μ mol/L FeSO + 100 μ mol/L H₂O₂.

The biochemical estimations were carried out by using the usual techniques (Tables 1 and 2).

In vivo: Detailed evaluation of extracts of *S. mukorossi* and *R. emodi* for hepatoprotective activity was carried out against CCl₄. The animals were divided into five groups of six animals each. Group 1 served as vehicle control and was administered with normal saline. Group 2 rats were given CCl₄ 1.0 mL/kg, *p.o* checking the biochemical parameters periodically for hepatotoxicity. Group 3 rats were given CCl₄ + extracts of *S. mukorossi* 2.5 g/kg, *p.o*. Group 4 rats were given CCl₄ + extracts of *R. emodi* 3.0 g/kg, *p.o*. Group 5 rats were given CCl₄ + Silymarin 50 g/kg, *p.o*. Blood was collected from the orbital sinus in all animals 2 h after last treatment and serum separated for different estimations (Table 3). The rats were anesthetized and sacrificed after the experimental period by cervical decapitation. The liver tissue was examined histopathologically.

Statistical analysis

The data obtained was subjected to statistical analysis using ANOVA for comparing different groups (Armitage, 1987) and Dunnett's *t* test for control and test groups (Dunnett, 1964). The two tailed unpaired student *t* test for comparing means before and after treatment and one

tailed unpaired student *t* test for comparing control and drug treated group, ED50 value with 95% confidence limits (CL) by regression analysis using log dose response (Swinscow, 1980 & Ghosh, 1984) were used. *P* < 0.05 or less was taken as the criterion of significance.

RESULTS

In vitro study

S. mukorossi and *R. emodi* extracts showed significant hepatoprotective activity against CCl₄ induced liver injury in primary hepatocytes cultures (Table 2). The hepatotoxic effects of CCl₄ are attributed to its metabolism by P450 to yield toxic trichloromethyl radicals that can act as free radical initiators^[28]. These radicals are believed to induce injury either by interacting with the unsaturated fatty acids of cell membranes, thereby causing lipid peroxidation, or by binding covalently to important macromolecules such as proteins, lipids, or DNA^[29,30]. The extracts of *S. mukorossi* and *R. emodi* reduced the levels of LDH and GPT released from CCl₄ injured rat hepatocytes into the medium in a concentration dependent manner, thus signifying their hepatoprotective activity.

In vivo study

In CCl₄ intoxicated rats, serum activities of AST, ALT, ALP, and bilirubin were increased significantly when compared to the control (Table 3). The CCl₄ treated group

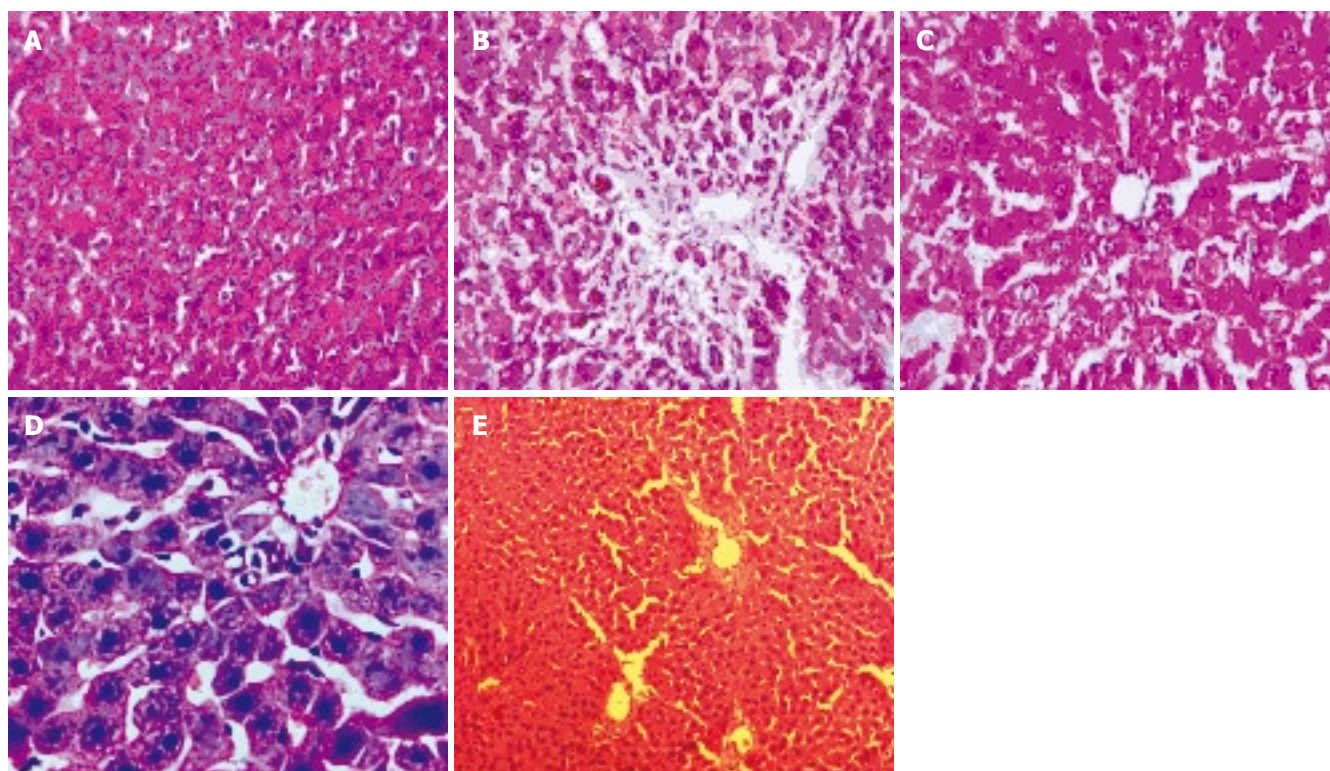


Figure 1 Photograph of rat liver shows (HE, $\times 100$). **A:** Liver of a control rat showing normal hepatocytes and normal architecture; **B:** Liver section from a CCl_4 treated rat demonstrating the destruction of architectural pattern, nodule formation in the lobular zone, inflamed periportal zone, moderate inflammation of portal area; **C:** Liver section from a Silymarin treated rat showing regeneration of normal hepatocytes; **D:** Liver section from a *R. emodi* treated rat showing normal lobular architecture; **E:** Liver section from a *S. mukorossi* treated rat showing normal lobular architecture no necrosis or fatty changes or any inflammatory reaction can be seen.

showed a marked increase in serum bilirubin (mg \%) (0.82 ± 0.08), ALT (IU/L) (1142.36 ± 80.97), AST (IU/L) (833.59 ± 34.85), and ALP (IU/L) (64.06 ± 3.00) activity indicating the injury caused by CCl_4 . Treatment with the extracts of *S. mukorossi* and *R. emodi* significantly decreased the above elevated parameters and the normal architectural liver pattern was restored as given below.

Liver section of control rat showed normal hepatocytes and normal architecture (Figure 1A). Liver sections from CCl_4 treated rats demonstrated the destruction of architectural pattern, nodule formation in the lobular zone, inflamed periportal zone, and moderate inflammation of portal area (Figure 1B). Liver sections from Silymarin treated rats showed regeneration of normal hepatocytes (Figure 1C). Liver sections from *R. emodi* treated rats showed normal lobular architecture (Figure 1D). Liver sections from *S. mukorossi* treated rat showed normal lobular architecture, and no necrosis or fatty changes or inflammatory reaction were seen (Figure 1E). These histopathological findings demonstrate a hepatoprotective effect of the extracts against CCl_4 -mediated liver damage.

DISCUSSION

The purpose of this study was to explore the hepatoprotective effect of *S. mukorossi* and *R. emodi* extracts in the hepatic damage caused by CCl_4 . Administration of CCl_4 to normal rats increased serum levels of AST, ALT, ALP, and bilirubin. The enzymes leaking out from damaged liver cells into circulating blood represent the damage to hepatic cells.

It is well established that the toxic metabolite of CCl_4 , a free radical CCl_3 is responsible for damage to liver cells. *S. mukorossi* and *R. emodi* extracts caused statistically significant decrease in all the above parameters at the dose of 2.5 mg/kg and 3.0 mg/kg given orally to CCl_4 treated rats. Histopathological examination of the liver sections of rats treated with CCl_4 showed destruction of architectural pattern, nodule formation in the lobular zone, inflamed periportal zone, moderate inflammation of portal area (Figure 1B). The group of rats treated with an extract of *R. emodi* showed normal lobular architecture (Figure 1D).

The group of rats treated with *S. mukorossi* showed normal lobular architecture and no necrosis or fatty changes or inflammatory reaction (Figure 1E). This suggests the reparative quality and maintenance of structural integrity of hepatocytic cell membrane of damaged liver cells by the extracts. The group of rats treated with Silymarin showing regeneration of normal hepatocytes was taken as standard (Figure 1C).

The ability of *S. mukorossi* and *R. emodi* to reduce the injurious effect or to preserve normal hepatic function disturbed by the hepatotoxin CCl_4 is the index of its hepatoprotective effect.

These findings show the prophylactic and curative efficacy of *S. mukorossi* and *R. emodi* in maintaining the integrity and functional status of hepatocytes.

In conclusion, the data presented here indicate that the extracts of *S. mukorossi* and *R. emodi* are hepatoprotective both in CCl_4 treated male rats and in CCl_4 treated cultured primary rat hepatocytes. In addition, the *in vivo* studies

carried out using the extracts also proved to be highly efficient in terms of dosage, tolerability, and restoring the liver. We now intend to look at the mechanism by which these extracts maintain the integrity of the liver.

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COMMENTS

Background

This article is based on the natural products responsible for repairing and healing of adversely affected liver cells. Our study suggests that the extracts of *S. mukorossi* and *R. emodi* are hepatoprotective both *in vitro* in CCl₄ damaged primary hepatocytes monolayer cultures and *in vivo* with doses of *S. mukorossi* (2.5 g/L) and *R. emodi* (3.0 g/L) in a CCl₄ induced liver injury animal model.

Research frontiers

Great effort has been and is still being done to minimize costs and side effects of synthetic drugs which are being used in the treatment of liver diseases.

Innovations and breakthroughs

The products are effective both *in vitro* and *in vivo* studies with minimal side effects and are cost effective. The herbal products were observed to have an excellent reparative effect on the CCl₄ damaged hepatocytes.

Applications

This article helps to understand and implement the process of treatment with herbal medicines in liver diseases, which is safe and cost effective in comparison with the synthetic drugs.

Peer review

This article tries to explore the safety and efficacy of the herbal drugs in the treatment of acute liver hepatitis and other liver disorders. The results revealed fewer side effects compared to synthetic drugs. It might be meaningful for the management and treatment of liver diseases in the clinic.

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RAPID COMMUNICATION

Ghrelin improves delayed gastrointestinal transit in alloxan-induced diabetic mice

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CONCLUSION: Ghrelin accelerates delayed GE and IT but has no effect on CT in diabetic mice. Ghrelin may exert its prokinetic effects via the cholinergic pathway in the enteric nervous system, and therefore has therapeutic potential for diabetic patients with delayed upper gastrointestinal transit.

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Key words: Ghrelin; Diabetes mellitus; Gastric emptying; Intestinal transit; Colonic transit

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Abstract

AIM: To investigate the effects of ghrelin on delayed gastrointestinal transit in alloxan-induced diabetic mice.

METHODS: A diabetic mouse model was established by intraperitoneal injection with alloxan. Mice were randomized into two main groups: normal mice group and diabetic mice group treated with ghrelin at doses of 0, 20, 50, 100 and 200 $\mu\text{g/kg}$ ip. Gastric emptying (GE), intestinal transit (IT), and colonic transit (CT) were studied in mice after they had a phenol red meal following injection of ghrelin. Based on the most effective ghrelin dosage, atropine was given at 1 mg/kg 15 min before the ghrelin injection for each measurement. The mice in each group were sacrificed 20 min later and their stomachs, intestines, and colons were harvested immediately. The amount of phenol red was measured. Percentages of GE, IT, and CT were calculated.

RESULTS: Percentages of GE, IT, and CT were significantly decreased in diabetic mice as compared to control mice (22.9 ± 1.4 vs 28.1 ± 1.3 , 33.5 ± 1.2 vs 43.2 ± 1.9 , 29.5 ± 1.9 vs 36.3 ± 1.6 , $P < 0.05$). In the diabetic mice, ghrelin improved both GE and IT, but not CT. The most effective dose of ghrelin was 100 $\mu\text{g/kg}$ and atropine blocked the prokinetic effects of ghrelin on GE and IT.

INTRODUCTION

Ghrelin is a 28-amino acid peptide that is synthesized in endocrine cells of the gastric mucosa^[1]. The major actions of this recently-discovered peptide include stimulation of growth hormone (GH) release^[1-3], regulation of appetite and nutrient ingestion^[4-6], and improvement of digestive motility^[7-9]. When injected into mice^[7,10], rats^[8,9], or dogs^[11], ghrelin accelerates gastric emptying after a liquid or solid meal. *In vitro* studies showed that in addition to known vagus nerve-dependent mechanisms, the activity of ghrelin is mediated via the enteric nervous system. Ghrelin increases electrically evoked cholinergic neural responses in strips of rat stomach^[12,13]. The presence of ghrelin and its receptors has been proven morphologically for myenteric neurons of guinea pig stomach and ileum^[14], but ghrelin receptors are difficult to identify on smooth muscles^[15].

Delayed gastrointestinal transit is a well-known diabetic complication, and may lead to uncomfortable gastrointestinal symptoms, such as frequent vomiting, emaciation, and unpredictable changes in blood glucose, all of which impair the quality of life of diabetic patients^[16,17]. Gastrointestinal transit of solid or nutrient liquid meals is abnormally slow in about 50% of diabetic patients^[16]. Delayed gastrointestinal transit may be associated

with cardiac autonomic neuropathy, blood glucose concentration, and gastrointestinal symptoms^[16].

Ghrelin has also been shown to accelerate gastric emptying in animal models of postoperative ileus^[18], septic ileus^[19], and burn-induced slow gastrointestinal transit^[19]. Ghrelin injections accelerate gastric emptying after a meal in humans even in the presence of deficient gastric innervation^[20]. However, ghrelin has never been studied in a diabetic animal model. It is unknown whether ghrelin can exert a similar prokinetic effect on the impaired gastrointestinal motility of diabetic mice, thus potentially having a clinical role in the treatment of impaired gastrointestinal motility in diabetic patients. In the present study, alloxan-induced diabetic mice were selected as an animal model of diabetic gastroparesis, resulting from autonomic neuropathy injury induced by prolonged hyperglycemia. We tested the effect of this newly-discovered gastric peptide on delayed gastrointestinal transit in diabetic mice.

MATERIALS AND METHODS

Chemicals

Rat ghrelin was obtained from Tocris Cookson (Bristol, UK). Atropine sulphate, phenol red, and alloxan were purchased from Sigma (St Louis, MO).

Diabetic mouse model

C57 mice (weighing 18–22 g) were provided by the Experimental Animal Center of Shanghai Academia Sinica. All procedures for the animal experiments were approved by the Medical Ethical Committee of Shanghai Jiaotong University. The mice were housed in stainless steel cages at a controlled temperature ($22 \pm 2^\circ\text{C}$) with a 60%–65% relative humidity in a normal 12-h light and dark cycle. After exposure to a high-fat diet for 3 wk, the mice were fasted overnight with free access to water and injected intraperitoneally with alloxan (0.2 g/kg body weight) dissolved in a sterile normal saline solution. Seventy-two hours later, the fasting blood glucose level in the mice was determined using the glucose oxidase method on a glucose analyzer. Mice with a blood glucose level higher than 11.1 mmol/L were classified as diabetic mice (DM mice). The DM mice were continuously fed without control of blood glucose for 4 wk, and a model of DM mice was established for further investigations.

Animal grouping for gastrointestinal transit studies

Gastric emptying, intestinal and colonic transit studies were then performed. In each study, mice were divided into two groups: a normal (control) group and a diabetic group. Mice in the diabetic group were treated with different doses of ghrelin (0, 20, 50, 100 and 200 $\mu\text{g/kg}$) given in a random order with a total of six mice in each subgroup. A dose-response curve for ghrelin was obtained for the experiment. Based on the most effective dose of ghrelin, another group of 6 mice was given atropine (1 mg/kg) injections 15 min before ghrelin injection.

Gastric emptying

After a 12-h fast, the diabetic mice were injected with

different doses of ghrelin (0, 20, 50, 100 and 200 $\mu\text{g/kg}$). After ghrelin injection, the mice received a gavage feeding (5 mg/kg body weight) of a phenol red test meal (0.5 g/L in 0.9% NaCl with 1.5% methylcellulose). Twenty minutes later, the mice were sacrificed. Their stomachs were clamped with a string above the lower oesophageal sphincter and a string beneath the pylorus to prevent leakage of phenol red. Gastric emptying was determined spectrophotometrically, according to the method previously described with certain slight modifications. The stomach of each individual mouse was resected just above the lower oesophageal sphincter and pyloric sphincter. Phenol red remained partly in the lumen of the stomach. The stomach and its contents were put into 5 mL of 0.1 mol/L NaOH. The stomach was minced. The samples containing the total amount of phenol red present in the stomach were further diluted to 10 mL with 0.1 mol/L NaOH and left at room temperature for 1 h. Five milliliters of the supernatant was then centrifuged at 800 g for 20 min. The absorbance was read at a wavelength of 546 nm on a spectrophotometer (Shanghai Yixian Company, China), and the phenol red content in the stomach was calculated. Percentage of gastric emptying of the phenol red was calculated as $[\text{infusion amount} - \text{remains}] / \text{infusion amount} \times 100\%$.

Intestinal and colonic transit

After an overnight fast, mice were given general anesthesia (2%–3% isoflurane inhalation) and underwent abdominal surgery. A small polyethylene tube was placed in the duodenum (or colon) *via* the stomach (or cecum), 0.5 cm distal to the pylorus (or ileocolic junction), fixed with sutures to the gut wall, and then tunneled through the abdominal wall subcutaneously to exit from the skin at the nape of the neck. Midline incisions were sutured, and mice were left to recover in separate cages. Food and water were abundantly provided. Three days later, after a 12 h fast, the mice were given different doses of ghrelin (0, 20, 50, 100 and 200 $\mu\text{g/kg}$) intraperitoneally. After ghrelin injection, a phenol red test meal at 5 mg/kg (0.5 g/L in 0.9% NaCl with 1.5% methylcellulose) was injected into the duodenum (or colon) *via* the implanted polyethylene tube. After 20 min, the mice were sacrificed. The distance of phenol red transit and the full length of the intestine or colon were calculated. Small intestine or colonic transit was assessed using the percentage ratio of phenol red transit over the full intestinal or colonic length.

Statistical analysis

Statistical analysis of the data was conducted using one-way ANOVA for multiple comparisons. Data are expressed as mean \pm SE. $P < 0.05$ was considered statistically significant.

RESULTS

Gastric emptying and intestinal and colonic transit in diabetic mice

Significantly delayed gastric emptying, intestinal and colonic transits were found in diabetic mice. Gastric emptying was significantly decreased in diabetic mice compared to normal mice ($22.9\% \pm 1.4\%$ *vs* $28.1\% \pm$

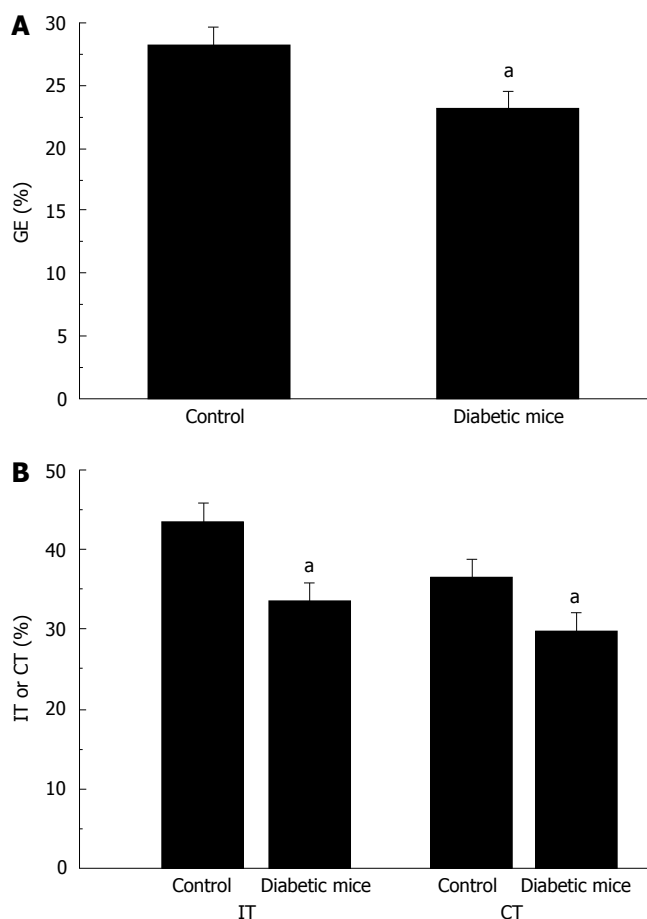


Figure 1 Delayed gastric emptying and intestinal and colonic transit in diabetic mice. **A:** Percentage of gastric emptying was significantly decreased in diabetic mice, ^a $P < 0.05$ vs control; **B:** Percentage of intestinal and colonic transits was significantly decreased in diabetic mice, ^a $P < 0.05$ vs control.

1.3%, $P < 0.05$, Figure 1A). Also, intestinal transit was significantly decreased in diabetic mice compared to normal mice ($33.5\% \pm 1.2\%$ vs $43.2\% \pm 1.9\%$, $P < 0.05$, Figure 1B). In addition, colonic transit was also decreased in diabetic mice compared to normal mice ($29.5\% \pm 1.9\%$ vs $36.3\% \pm 1.6\%$, $P < 0.05$, Figure 1B).

Effect of ghrelin on delayed gastric emptying in diabetic mice

Ghrelin significantly accelerated gastric emptying in diabetic mice. The percentage of gastric emptying was $24.3\% \pm 2.1\%$, $27.8\% \pm 1.0\%$, $34.5\% \pm 1.2\%$, and $32.9\% \pm 1.1\%$, respectively, in the mice treated with 20, 50, 100, and 200 $\mu\text{g}/\text{kg}$ ghrelin. Except for the lowest dose, all of these doses normalized the rate of gastric emptying in diabetic mice ($P < 0.05$, Figure 2A). We considered that 100 $\mu\text{g}/\text{kg}$ ghrelin could most effectively increase the rate of gastric emptying.

Effect of ghrelin on delayed small intestinal transit in diabetic mice

Ghrelin significantly accelerated intestinal transit in diabetic mice. The percentage of intestinal transit was $34.6\% \pm 2.0\%$, $38.2\% \pm 1.6\%$, $41.5\% \pm 1.9\%$, and $39.5\% \pm 1.8\%$, respectively, in the mice treated with 20, 50, 100, and 200 $\mu\text{g}/\text{kg}$ ghrelin. All of these doses except for 20 $\mu\text{g}/\text{kg}$

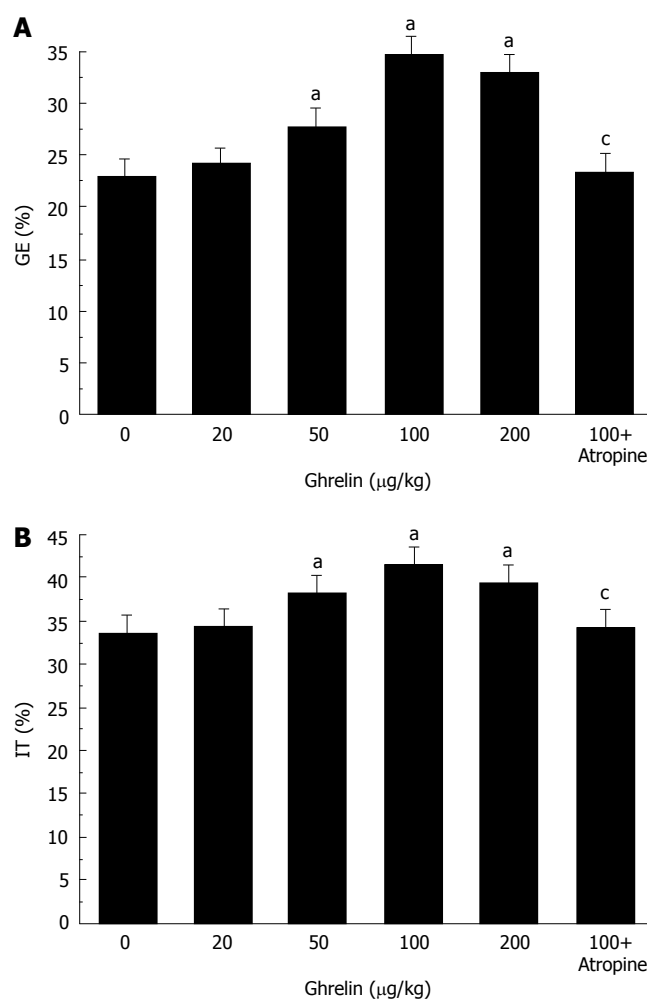


Figure 2 Ghrelin significantly increases gastric emptying (A) and intestinal transit (B) in diabetic mice. ^a $P < 0.05$ vs control, ^c $P < 0.05$ vs 100 $\mu\text{g}/\text{kg}$ ghrelin.

normalized the delayed intestinal transit ($P < 0.05$, Figure 2B). As above, 100 $\mu\text{g}/\text{kg}$ ghrelin was considered most effective in accelerating intestinal transit.

Effect of ghrelin on delayed colonic transit in diabetic mice

Ghrelin had no effect on delayed colonic transit. The percentage of colonic transit was $30.6\% \pm 1.3\%$, $30.2\% \pm 1.8\%$, $29.2\% \pm 1.5\%$, and $31.6\% \pm 2.2\%$, respectively, in the mice treated with 20, 50, 100, and 200 $\mu\text{g}/\text{kg}$ ghrelin. None of these doses was able to accelerate intestinal transit (Figure 3).

Effect of atropine on delayed gastric emptying and intestinal transit in diabetic mice

Atropine blocked the positive effect of 100 $\mu\text{g}/\text{kg}$ ghrelin on gastric emptying and intestinal transit. The percentage of gastric emptying was significantly decreased from $34.5\% \pm 1.2\%$ to $23.5\% \pm 1.7\%$ ($P < 0.05$, Figure 2A). The small intestine transit was significantly decreased from $41.5\% \pm 1.9\%$ to $34.2\% \pm 1.6\%$ ($P < 0.05$, Figure 2B).

DISCUSSION

In the present study, mice with alloxan-induced diabetes

had mild or moderate gastroparesis with slow gastric emptying and intestinal transit due to an autonomic neuropathic injury induced by prolonged hyperglycemia as compared with normal controls. Thus, mice with alloxan-induced diabetics could be used as an animal model of diabetic gastroparesis. Significantly delayed gastric emptying, intestinal and colonic transit were seen in the mice with alloxan-induced diabetics. Different doses of ghrelin were able to accelerate gastric emptying and intestinal transit in the diabetic mice, but had no effect on colonic transit. The most effective dose of ghrelin for accelerating upper gastrointestinal transit was 100 $\mu\text{g/kg}$ per mouse. Atropine blocked the ghrelin effect on gastric emptying and intestinal transit.

In the present study, gastric emptying and intestinal and colonic transit were significantly delayed in the mice with alloxan-induced diabetics, which is consistent with the previously reported findings^[21,22]. Gastrointestinal motility disturbances including esophageal motor dysfunction, gastroparesis, constipation and diarrhea, are common in patients with diabetes mellitus. It was reported that gastrointestinal transit is significantly slower in the diabetic animal model of human diabetes^[21-23]. Inhibition of gastrointestinal motility has also been reported in humans with diabetes mellitus^[24,25]. The pathogenesis of slow gastrointestinal transit in diabetes mellitus patients is not clear, but several mechanisms have been proposed^[16]. Among them, autonomic neuropathy, a complication of long-standing diabetes mellitus, has been widely accepted as the culprit. This may lead to the absence of postprandial gastrointestinal response, a reflex that should present in healthy people^[24]. Recent studies have shown that an acute change in blood glucose concentration also has a major effect on gastrointestinal motor function in healthy subjects^[25]. In particular, acute hyperglycemia inhibits both the gastrointestinal and ascending components of peristaltic reflex. Poor glycemic control has the potential to cause delayed gastrointestinal transit in diabetic patients^[26].

To the best of our knowledge, this is the first report on the effect of ghrelin on gastrointestinal dysmotility in diabetic mice. Ghrelin possessing prokinetic characteristics increases gastric emptying in healthy mice^[27,28], gastric emptying^[13,29,30], frequency of migrating motor complex, and intestinal transit^[31,32] in healthy rats. In healthy dogs, ghrelin stimulates antral contractility and antroduodenal coordination, thus increasing gastric emptying^[33]. In healthy volunteers^[29], subjects with normal weight^[34], and gastroparetic^[35] human subjects with gastroparesis, ghrelin also increases gastric emptying. The mechanisms underlying the action of ghrelin seem to be neuron-dependent. *In vitro*, isolated strips of muscle fail to contract significantly when exposed to ghrelin. *In vivo*, the gastrokinetic effect of ghrelin in rats is abolished by atropine and vagotomy. Diabetic gastroparesis is classically attributed to autonomic neuropathy induced by prolonged hyperglycemia, which did not preclude the gastrokinetic effect of ghrelin in our investigation. Our data strongly suggest that ghrelin can exert a prokinetic action on the upper alimentary tract *via* the enteric nervous pathway. In our study, atropine blocked the effect of 100 $\mu\text{g/kg}$ ghrelin

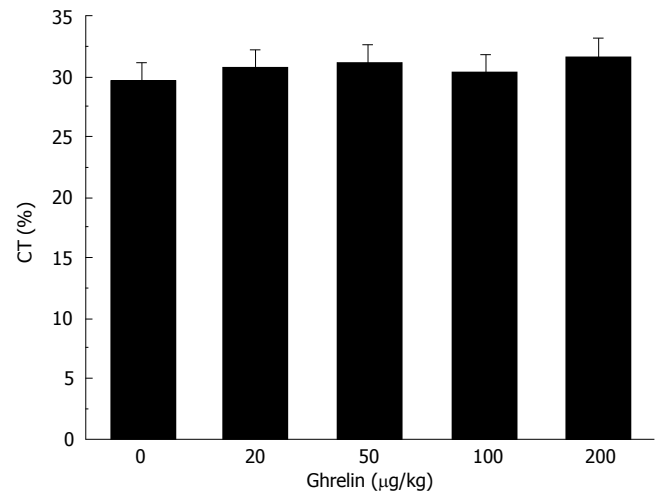


Figure 3 No effect of Ghrelin on delayed colonic transit in diabetic mice.

on gastric emptying and intestinal transit, suggesting that the prokinetic effect of ghrelin is mediated *via* the cholinergic pathway in the enteric nervous system. There are other mechanisms involving tachykinergic pathways, as demonstrated in electrical field stimulation studies in isolated rat stomach^[13].

Moreover, we found that the dose-response relationships of ghrelin between gastric emptying and colonic transit in diabetic mice were bell-shaped. There are many causes for bell-shaped dose-response curves. Desensitization is one of the possibilities, because ghrelin receptor is susceptible to rapid desensitization^[36]. Another possibility is the existence of high and low affinity receptor binding sites in different pathways. In our study, however, ghrelin showed no effect on colon transit, which is consistent with the results seen both in animal models of postoperative ileus^[18] and in burn-induced gastrointestinal delayed transit^[19]. We believe that this result might be related to the distribution of ghrelin receptors in the gut^[37,38].

Based on this study, it is reasonable to assume that ghrelin can be used as a potential drug for the treatment of diabetic patients with delayed gastrointestinal transit. Clinically, improvement in gastrointestinal transit facilitates enteral resuscitation, corrects blood glucose concentrations and reduces gastrointestinal symptoms in diabetic patients.

In conclusion, ghrelin accelerates gastric emptying and intestinal transit in diabetic mice, an action that may be mediated *via* the cholinergic pathway in the enteric nerve system. Ghrelin may have a therapeutic potential for diabetic patients with delayed upper gastrointestinal transit. The physiological role of ghrelin in the gastrointestinal tract remains to be identified, and its pharmacotherapeutic potential deserves to be further explored in diabetic patients suffering from delayed upper gastrointestinal transit.

COMMENTS

Background

Delayed gastrointestinal transit is common in patients with chronic diabetes and is always associated with impairments in quality of life and diabetic control. Ghrelin is

a potent prokinetic peptide. The effect of ghrelin on delayed gastrointestinal transit was studied in diabetic mice.

Research frontiers

The effects of ghrelin on gastrointestinal motor activity and roles in motility regulation have been extensively studied. This study is the first to investigate the effects of ghrelin on diabetic mice with delayed gastrointestinal transit.

Innovations and breakthroughs

Ghrelin has been shown to accelerate gastric activity in postoperative and septic ileus animal models. However, it has not been studied in a diabetic animal model.

Applications

According to animal experiments, ghrelin may be used as a potential therapeutic agent for the treatment of delayed gastrointestinal transit in diabetes.

Peer review

This paper shows for the first time that ghrelin has an effect on gastric emptying and intestinal transit in diabetic mice, which may be mediated through the cholinergic pathways in the enteric nerve system. These results show that ghrelin can be used as a potential therapeutic drug for the treatment of delayed gastrointestinal transit in diabetes.

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RAPID COMMUNICATION

Effect of histone deacetylase inhibitor on proliferation of biliary tract cancer cell lines

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Abstract

AIM: To explore the effect of histone deacetylase inhibitor, trichostatin A (TSA) on the growth of biliary tract cancer cell lines (gallbladder carcinoma cell line and cholangiocarcinoma cell line) *in vivo* and *in vitro*, and to investigate the perspective of histone deacetylase inhibitor in its clinical application.

METHODS: The survival rates of gallbladder carcinoma cell line (Mz-ChA-I cell line) and cholangiocarcinoma cell lines (QBC939, KMBC and OZ cell lines) treated with various doses of TSA were detected by methylthiazol tetrazolium (MTT) assay. A nude mouse model of transplanted gallbladder carcinoma (Mz-ChA-I cell line) was successfully established, and changes in the growth of transplanted tumor after treated with TSA were measured.

RESULTS: TSA could inhibit the proliferation of gallbladder carcinoma cell line (Mz-ChA-I cell line) and cholangiocarcinoma cell lines (QBC939, KMBC and OZ cell lines) in a dose-dependent manner. After the nude mouse model of transplanted gallbladder carcinoma (Mz-ChA-I cell line) was successfully established, the growth of cancer was inhibited in the model after treated with TSA.

CONCLUSION: TSA can inhibit the growth of cholangiocarcinoma and gallbladder carcinoma cell lines *in vitro* and *in vivo*.

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Key words: Biliary tract cancer; Gallbladder carcinoma; Cholangiocarcinoma; Proliferation; Trichostatin A

INTRODUCTION

Biliary tract cancer, consisting of gallbladder carcinoma and cholangiocarcinoma, presents many challenges to physicians. It is a relatively rare cancer often causing a diagnostic dilemma, as its presentation may be similar to that of non-malignant conditions^[1,2]. It was reported that the misdiagnosis rate of biliary duct cancer is 19.1% and the median survival time of biliary tract cancer patients is about 7 mo^[3]. At present, treatment of biliary tract cancer is also difficult^[4] and complex due to a morbid patient population and limited data on the optimal therapeutic approach. Surgery remains the mainstay of treatment, although the extent of resection required is still controversial. Despite recent advances in imaging modalities, most of the patients are at the advanced stage of the disease at presentation, thus making radical surgery not feasible, which seriously affects its prognosis. The role of adjuvant therapy is also controversial. Different chemotherapeutic regimens have been investigated in small uncontrolled studies, with generally disappointed results^[5]. In patients with unresectable biliary duct cancer, combination of chemotherapy and radiotherapy can result in a prolonged survival time of some patients. In a palliative setting, biliary stenting and other supportive measures can alleviate symptoms and improve survival. Ultimately, treatment decisions should be individualized and participation in clinical trials is encouraged. Further progress in the management of biliary tract cancer is anticipated using biological therapies and continued research is essential to discover the optimal treatment for this challenging disease^[2]. It is, thus, important to research its biological characteristics and biotherapy.

Cancer is a disease resulting from both genetic and epigenetic changes. There are many important epigenetic changes in its early carcinogenesis. Accumulating evidence indicates, however, that disparities in gene expression resulting from variable modifications in DNA methylation and chromatin structure in response to the environ-

ment also play a role in differential susceptibility to the disease^[6,7]. Histone acetylation/deacetylation constitutes the most relevant chromatin remodelling mechanism underlying the DNA access to nuclear machinery and mutagenic agents^[8]. It has been recently shown that histone acetylation/deacetylation is closely related with tumorigenesis^[9,10]. Acetylation of histone plays a very important but incompletely understood role in genetic regulation^[11]. Histone deacetylase inhibitors could markedly inhibit the growth of tumor cell lines^[12,13]. It has been shown that expression of p53BP1, a non-histone protein, is associated with HDAC4 and plays a role in histone acetylation of fully-grown oocytes^[14]. Other data indicate that the known non-histone targets may play a role in the pathogenesis of cholangiocarcinoma^[15].

Experiments *in vitro* have shown that TSA can be used in the treatment of ovarian cancer, pancreatic endocrine carcinoma, prostate cancer, *etc*^[16-18]. The results of this study focusing on the effect of histone deacetylase inhibitor, trichostatin A (TSA) on the proliferation of gallbladder carcinoma cell line (Mz-ChA-I cell line) and cholangiocarcinoma cell lines (QBC939, KMBC and OZ cell lines) *in vivo* and *in vitro*, have demonstrated the value of biotherapy for biliary tract cancer with TSA.

MATERIALS AND METHODS

Cell lines

QBC939 cell line (a human cholangiocarcinoma cell line), KMBC cell line (a human cholangiocarcinoma cell line), OZ cell line (a human cholangiocarcinoma cell line) and Mz-ChA-I cell line (originally isolated from human gallbladder adenocarcinoma) were used in our study. QBC939 cell line was kindly provided by Professor Shu-Guang Wang, The Third Military Medical University, Chongqing, China. KMBC, OZ and Mz-ChA-I cell lines were kindly provided by Dr. Ke-Qin Hu, University of California, Irvine Medical Center, California, USA. Cell culture media and supplements were purchased from GIBCO Invitrogen Corporation (Carlsbad, CA, USA).

Animals

Sixteen male BALB/c nude mice at the age of 4 wk, weighing 10.0 ± 2.1 g, were purchased from the Experimental Animal Center of Tongji Medical College, Huazhong University of Science and Technology (Wuhan, China). The mice were caged individually under specific pathogen-free conditions and fed with standard maintenance diet and water throughout the experimental period. All animals received care treatment in compliance with the Guidelines of Ministry of Public Health of China.

Cell culture

All biliary tract cancer cell lines (QBC939, KMBC, OZ and Mz-ChA-I cell lines) were grown as a monolayer in RPMI-1640 medium (Sigma, USA) supplemented with 10% fetal bovine serum, penicillin (100 000 U/L), cultured in T-75 cm² culture flasks, maintained at 37°C in a humidified atmosphere containing 50 mL/L CO₂. At the beginning of experiment, cells at the exponential growth phase were removed from the flask with a solution containing

0.25% trypsin and 0.02% EDTA and seeded in 96-well plates containing RPMI-1640 medium supplemented with 10% fetal bovine serum.

Methylthiazol tetrazolium (MTT) assay

QBC939, KMBC, OZ and Mz-ChA-I cells at logarithmic growth phase were digested with 0.25% trypsin and then suspended. Cells were calculated under microscope with the cell suspension adjusted to 2×10^5 /mL. The wells in the plate were divided into 6 experiment groups and 1 blank control group. Cells were added into the plate (200 μ L per well, 0.4×10^5), and medium into the blank control wells. After 18 h, the medium for the experiment groups was replaced with a medium containing 0.10, 0.25, 0.50, 1.0, 1.5, 2.0 μ mol/L TSA, respectively. The culture was continued, then 20 μ L of 5 mg/mL MTT was added into each well after 4, 12, 24, 36 and 48 h, respectively. After another 4 h, the medium was discarded and 150 μ L DMSO was added into each well. The *A* value for each well was measured at the wave length of 490 nm. The survival rate was then calculated and the survival curve was plotted following the formula: survival rate (%) = the *A* value of experiment groups - the *A* value of blank control group / the *A* value of negative group - the *A* value of blank control group $\times 100\%$.

Treatment of Mz-ChA-I cell lines and establishment of gallbladder carcinoma transplanted tumor model

Mz-ChA-I cells at the logarithmic phase were divided into two groups: one group was treated with 0.75 μ mol/L TSA, the other group was cultured in medium. Cells were digested and counted after 24 h. The 16 male BALB/c nude mice at the age of 4 wk were randomly divided into group A (control group) and group B (TSA group) randomly, 8 in each group. The mice in group A were subcutaneously inoculated with untreated Mz-ChA-I cell suspension *via* the back, while those in group B were subcutaneously inoculated with Mz-ChA-I cell suspension treated with TSA for 24 h *via* the back. The wound was slightly pressed with a cotton swab for hemostasis and closed. The volume of cell suspension was 0.2 mL, with a concentration of 1×10^5 /mL. The volume of tumors was calculated in the sixth week according to the following formula: the volume = the biggest diameter \times transverse diameter²/2.

Statistical analysis

Statistical analysis was performed using the rank sum test (*H* test) for multi-sample comparison and *t*-test for two-sample comparison. The data were expressed as mean \pm SE. *P* < 0.05 was considered statistically significant.

RESULTS

Effect of TSA on survival of QBC939, KMBC, OZ and Mz-ChA-I cell lines *in vitro*

QBC939, KMBC, OZ and Mz-ChA-I cell lines were shrunk and died when TSA was added at various concentrations. MTT assay showed that TSA could shorten the survival time of QBC939, KMBC, OZ and Mz-ChA-I cell lines in a dose-dependent manner. The inhibition of cell survival increased with the increased dose of TSA (Table 1, Figure 1).

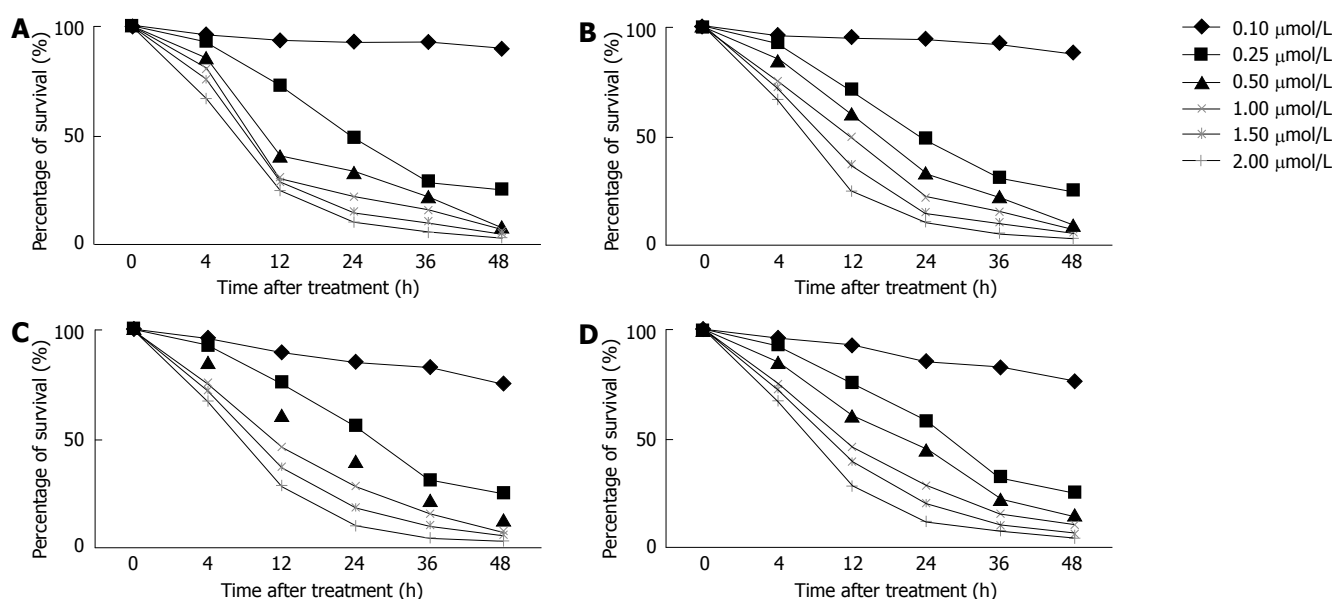


Figure 1 Effect of TSA on the survival of QBC₉₃₉ (A), KMBC (B), OZ (C), and Mz-ChA-I (D).

Table 1 Effect of TSA on the survival of biliary tract cancer cell lines

Concentration of TSA	Percentage of survival of cell lines (median and quartile)			
	QBC ₉₃₉	KMBC	OZ	Mz-ChA-I
0.10 μmol/L	92.87 (93.09)	93.27 (94.87)	83.07 (87.72)	83.26 (88.75)
0.25 μmol/L	33.38 (60.61)	34.88 (59.60)	36.63 (65.60)	38.58 (66.51)
0.50 μmol/L	24.90 (36.45)	24.86 (46.64)	26.90 (50.64)	27.90 (52.64)
1.00 μmol/L	16.99 (26.30)	17.09 (35.80)	18.46 (37.23)	18.46 (37.23)
1.50 μmol/L	11.29 (21.63)	11.32 (25.36)	12.29 (27.36)	12.79 (29.86)
2.00 μmol/L	6.52 (17.35)	6.28 (16.36)	6.28 (19.25)	9.03 (20.25)
Value of <i>H</i>	15.66	16.11	14.23	14.32
<i>P</i>	0.0082	0.0076	0.046	0.043

The survival percentage of cell lines treated with various doses of TSA was detected after 4, 12, 24, 36 and 48 h, respectively, by MTT assay. The MTT value was expressed as median and quartile.

Effect of TSA on survival of Mz-ChA-I cell line

All the male BALB/c nude mice survived. The mice in group A were subcutaneously inoculated with untreated Mz-ChA-I cell suspension *via* the back. The mice in group B were subcutaneously inoculated with Mz-ChA-I cell suspension *via* the back after treated with TSA for 24 h. After the Mz-ChA-I cell line was transplanted into the nude mice for 6 wk, the tumor volume of group A was $930.25 \pm 261.64 \text{ mm}^3$, the transplanted tumor volume of group B was smaller than that of group A, and there was a significant difference between them ($t = 2.50$, $P = 0.036$).

DISCUSSION

Regulation of genes is a new topic^[19]. Genetic information is regulated and expressed precisely. Gene expression can be greatly regulated by histone remodeling. This is a kind of basic post formation theory of gene regulation^[20]. Histone deacetylase (HDAC) and histone acetyl transferase (HAT) are two counteracting enzymes. Their activities can control the acetylation of protein lysine residues, notably those contained in the N-terminal extensions of the core

histones. Acetylation of histones affects gene expression through its influence on chromatin conformation^[21,22]. A lot of data have shown that HDAC is one of the promising targets of cancer treatment as many HDAC inhibitors of solid and liquid tumors have entered clinical trials^[23]. HDAC inhibitor is a kind of chemical compound regulating gene expression at the transcription level by changing chromosome structure through inhibiting HDAC. The HDAC inhibitors-inhibited HDAC enzymes shift the balance between the deacetylation activity of HDAC enzymes and the acetylation activity of histone acetyltransferases, resulting in hyperacetylation of core histones. Exposure of cancer cells to HDAC inhibitors is associated with a multitude of molecular and biological effects, ranging from transcriptional control, chromatin plasticity, protein DNA interaction to cellular differentiation, growth arrest and apoptosis. HDAC inhibitors are an exciting new addition to the arsenal of cancer therapeutics^[24,25].

This study focused on the effect of histone deacetylase inhibitor, trichostatin A (TSA) on the survival of QBC939, KMBC, OZ and Mz-ChA-I cell lines and determined if TSA can be used in the treatment of biliary tract cancer. Our results show that TSA could shorten the survival time of QBC939, KMBC, OZ and Mz-ChA-I cell lines *in vitro* in a dose-dependent manner. We have successfully established a nude mouse model of transplanted gallbladder carcinoma. The growth of biliary tract cancer was inhibited in the mice after treatment with TSA. Our results show that TSA could shorten the survival time of gallbladder carcinoma and cholangiocarcinoma cell lines *in vivo* and *in vitro*, indicating that TSA is a potential drug for the treatment of biliary tract cancer. It was reported that HDACIs MS-275, NVP-LBH589 and NVP-LAQ824 can effectively inhibit the growth of human biliary tract cancer cells^[26,27]. However, early diagnosis and treatment of biliary tract cancer are still difficult^[29-34]. In order to improve its diagnosis and treatment, further study on its epidemiology, clinicopathology and molecular biology is needed.

COMMENTS

Background

Biliary tract cancer, consisting of gallbladder carcinoma and cholangiocarcinoma, presents many challenges to physicians. At present, treatment of biliary tract cancer is difficult. This study focused on the effect of trichostatin A (TSA) on the proliferation of biliary tract cancer cells.

Research frontiers

Cancer is a disease resulting from both genetic and epigenetic changes. There are many important epigenetic changes in its early carcinogenesis. It has been recently shown that histone acetylation/deacetylation is closely related with tumorigenesis. Acetylation of histone plays a very important but incompletely understood role in genetic regulation. Histone deacetylase inhibitors could markedly inhibit the growth of tumor cells.

Innovations and breakthroughs

TSA can inhibit the growth of cholangiocarcinoma and gallbladder carcinoma cell lines *in vitro* and *in vivo*.

Applications

TSA can be used as a potential drug for the treatment of biliary tract cancer.

Peer review

This manuscript describes the effect of histone deacetylase inhibitor on the proliferation of four biliary tract cancer cell lines. The study was well designed, and had novel findings. The conclusion is of clinical value.

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RAPID COMMUNICATION

Increased N-terminal pro-brain natriuretic peptide level predicts atrial fibrillation after surgery for esophageal carcinoma

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Abstract

AIM: To evaluate the value of plasma N-terminal pro-brain natriuretic peptide (NT-proBNP) level for predicting postoperative atrial fibrillation (AF) in patients undergoing surgery for esophageal carcinoma.

METHODS: NT-proBNP levels were measured in 142 patients 24 h before and 1 h after surgery for esophageal carcinoma. All patients having a preoperative cardiac diagnosis by electrocardiogram (ECG), remained under continuous monitoring for at least 48 h after surgery, and then underwent clinical cardiac evaluation until discharge.

RESULTS: Postoperative AF occurred in 11 patients (7.7%). AF patients were significantly older (69.6 ± 12.2 years *vs* 63.4 ± 13.3 years, $P = 0.031$) than non-AF patients. There were no significant differences in history of diabetes mellitus, sex distribution, surgical approach, anastomosis site, intraoperative hypotension and postoperative fever. The preoperative plasma NT-proBNP level was significantly higher in patients who developed postoperative AF (121.3 ± 18.3 pg/mL *vs* 396.1 ± 42.6 pg/mL, $P = 0.016$). After adjustment for age, gender, chronic obstructive pulmonary disease (COPD), history of cardiac diseases, hypertension, postoperative hypoxia and thoracic-gastric dilation, NT-proBNP levels were found to be associated with the highest risk factor for

postoperative AF (odds ratio = 4.711, 95% CI = 1.212 to 7.644, $P = 0.008$).

CONCLUSION: An elevated perioperative plasma BNP level is a strong and independent predictor of postoperative AF in patients undergoing surgery for esophageal carcinoma. This finding has important implications for identifying patients at higher risk of postoperative AF who should be considered for preventive antiarrhythmic therapy.

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Key words: Esophageal carcinoma; Atrial fibrillation; Natriuretic peptides; Surgery

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INTRODUCTION

Postoperative infections and cardiac events are the major complications after surgery for esophageal carcinoma and the dominating causes of death. Atrial fibrillation (AF) is a frequent arrhythmia after esophageal procedures and is associated with an increased morbidity and mortality and a longer, more expensive hospital stay^[1-5].

In a previous study, we retrospectively studied 63 patients with AF after surgery for esophageal carcinoma in comparison with 126 patients without AF after esophagectomy during the same time. We identified some risk factors as predictors of AF after surgery for esophageal carcinoma, such as postoperative hypoxia, history of obstructive pulmonary disease (COPD), thoracic-gastric dilatation, age older than 65 years, male gender, and history of cardiac disease^[6]. However, our ability to accurately identify patients at high risk for AF is still limited. No accurate assessment tool or biomarker has

been identified that could predict the occurrence of AF early after esophageal procedures so far. Since a targeted preventive treatment cannot be performed easily, a sensitive blood biomarker that can predict the occurrence of AF in patients after surgery for esophageal carcinoma with a high specificity is desirable.

Brain natriuretic peptide (BNP) is a neurohormone which is stored mainly in myocytes of the cardiac ventricles and released as a result of volume and pressure overload or myocardial damage^[7-10]. N-terminal pro BNP (NT-proBNP) has a longer half life than brain natriuretic peptide and is less influenced by acute therapeutic regimens and clinical deteriorations, making it available for predicting cardiac functions^[11-14]. NT-proBNP has been proved useful for diagnostic and prognostic purposes in patients with congestive heart failure and other cardiac conditions^[15-18]. It was recently reported that increased NT-proBNP can indicate the underlying subclinical predisposition to AF both in patients undergoing cardiac surgery^[19] and in patients without a history of cardiac disease^[20]. To our knowledge, the ability of NT-proBNP to predict AF after esophageal procedures has not been evaluated.

We speculate that an elevated level of NT-proBNP could predict the occurrence of AF early after esophageal procedures. The aim of this study is to determine whether NT-proBNP levels are associated with AF after surgery for esophageal carcinoma.

MATERIALS AND METHODS

Patients

One hundred and fifty consecutive patients undergoing elective surgery for esophageal cancer were identified in our hospital from December 2006 to May 2007. Patients with a history of heart failure ($n = 3$), chronic AF ($n = 2$), severe renal dysfunction ($n = 1$), and antiarrhythmic drug use ($n = 2$) were excluded from the study. One hundred and forty-two patients (113 males and 29 females with an average age of 66.5 years, range 49-86 years) accordant with the inclusion criteria were enrolled in the study.

All patients having a preoperative cardiac diagnosis by electrocardiogram (ECG) remained under continuous monitoring for at least 48 h after surgery and then underwent clinical cardiac evaluation until discharge. AF was defined as absent P wave before the QRS complex with irregular ventricular rhythm on the rhythm strips.

Plasma NT-proBNP concentration was measured 24 h before and soon (within 1 h) after surgery. Blood samples were collected into tubes containing potassium EDTA, centrifuged for 5 min at 1500 r/min and kept at 4°C. The separated plasma was kept at -30°C until analysis. NT-proBNP analyses were done with Elecsys Roche Diagnostics commercial kits on a semiautomatic analyzer (Elecsys-2010, Roche Diagnostics, Germany). The test is self-processing and produces a result within 15 min. The precision, analytic sensitivity and stability characteristics of this system have been described elsewhere^[21].

Statistical analysis

Continuous variables are expressed as mean \pm SD or median (range) and were compared by Student's *t*-test.

Chi square test or Fisher's exact test was used to compare groups of categorical data. The relationship between the occurrence of postoperative AF and baseline predictors was assessed with multivariable logistic regression model adjusted for factors presumably associated with AF risk, including advanced age, male gender, COPD, cardiac diseases, hypertension and diabetes mellitus, site of anastomosis, postoperative hypoxia, thoracic-gastric dilation and plasma NT-proBNP^[1,22-25]. $P < 0.05$ was considered statistically significant. Statistical analysis was performed using the SPSS 11.0 statistical software package (SPSS, Inc).

RESULTS

The baseline characteristics of the 142 patients are listed in Table 1. The patients were divided into 2 groups according to whether they developed postoperative AF. Postoperative AF occurred in 11 patients (7.7%). Postoperative AF patients were significantly older (69.6 ± 12.2 years *vs* 63.4 ± 13.3 years, $P = 0.031$) than non-postoperative AF patients. Patients in the postoperative AF group had a history of COPD (45.5% *vs* 12.2%, $P = 0.021$) and cardiac disease more frequently (6.9% *vs* 27.3%, $P = 0.046$). Postoperative AF patients had a significantly higher incidence of postoperative hypoxia (36.4% *vs* 8.4%, $P = 0.018$) and thoracic-gastric dilation (45.5% *vs* 12.2%, $P = 0.021$) than non-postoperative AF patients. There were no significant differences in gender, history of hypertension, diabetes mellitus, surgical approach, anastomosis site, intraoperative hypotension and postoperative fever between the two groups. Preoperative plasma NT-proBNP level was significantly higher in patients who developed postoperative AF (121.3 ± 18.3 pg/mL *vs* 396.1 ± 42.6 pg/mL, $P = 0.016$).

In a multivariable logistic regression model adjusted for age, gender, COPD, history of cardiac disease, hypertension, diabetes mellitus, site of anastomosis, postoperative hypoxia, thoracic-gastric dilatation and plasma NT-proBNP, NT-proBNP levels were associated with the highest risk factor for postoperative AF (odds ratio = 4.711, 95% CI = 1.212 to 7.644, $P = 0.008$) (Table 2).

DISCUSSION

AF after esophagectomy remains one of the most frequently encountered complications. The incidence of AF in this study was 7.7%, lower than the reported data ranging from 11.3% to 22%^[26,27]. Although the causes for postoperative AF after esophagectomy have not been completely disclosed, we speculate that AF after esophagectomy is precipitated by the resolution of inflammatory response following blunt or sharp surgical trauma to sympathovagal nerve fibers supplying the heart, which alters the autonomic modulation of atrial myocardial cells to endogenous catecholamines. Although postoperative AF is self-limited in most cases, it can increase the risk of postoperative stroke^[28]. The treatment of postoperative AF requires a prolonged hospital stay and additional costs. Although previous studies^[6,22,27] have shown different risk factors for postoperative AF,

Table 1 Characteristics of study subjects, *n* (%)

Characteristic	No postoperative AF (<i>n</i> = 131)	Postoperative AF (<i>n</i> = 11)	<i>P</i>
Age (yr)	63.4 ± 13.3	69.6 ± 12.2	0.031
Male	109 (83.2)	9 (81.8)	0.971
COPD	16 (12.2)	5 (45.5)	0.021
History of cardiac diseases	9 (6.9)	3 (27.3)	0.046
History of hypertension	8 (6.1)	1 (9.1)	0.717
Diabetes mellitus	7 (5.3)	1 (9.1)	0.63
Site of anastomosis			
Neck	28 (21.4)	3 (27.3)	0.721
Above the aortic arch	81 (61.8)	6 (54.5)	0.812
Below the aortic arch	22 (16.8)	2 (18.2)	0.921
Right thorax approach	12 (9.2)	1 (9.1)	0.994
Intraoperative hypotension	9 (6.9)	1 (9.1)	0.798
Postoperative fever	29 (22.1)	3 (27.3)	0.76
Postoperative hypoxia	11 (8.4)	4 (36.4)	0.018
Thoracic-gastric dilatation	16 (12.2)	5 (45.5)	0.021
NT-proBNP, pg/mL			
Before surgery	121.3 ± 18.3	396.1 ± 42.6	0.016
After surgery	160.3 ± 17.3	589.5 ± 51.2	0.009

a definite relation between these factors and occurrence of AF has not been well established. A proper preventive treatment of AF is still a challenge. Even if preventive therapies with antiarrhythmic agents can reduce the occurrence of postoperative AF, their use has been limited because of potential side effects^[29].

BNP and NT-proBNP are members of the natriuretic peptide family synthesized and secreted by the ventricular myocardium. The natriuretic peptide family plays a role in regulation of the cardiovascular system^[30]. Moreover, it was reported that an elevated NT-proBNP level can be used as a diagnostic and prognostic marker in patients with congestive heart failure, myocardial infarction, unstable angina and left ventricular hypertrophy^[15-18].

The present study evaluated the role of NT-proBNP in predicting postoperative AF in patients undergoing surgery for esophageal carcinoma. The results showed that an elevated level of plasma NT-proBNP obtained before or soon after surgery for esophageal carcinoma was a strong and independent predictor of the occurrence of postoperative AF, suggesting that patients can be stratified according to their risk of postoperative AF. A targeted preventive therapy may be performed only for high risk patients with an elevated NT-proBNP level before or soon after esophageal surgery to prevent the occurrence of operative AF. Low-risk patients with a high negative predictive value of NT-proBNP should not receive any preventive treatment.

Our study provided a new prospective in terms of preventive strategies against postoperative AF. It was reported that NT-proBNP-tailored therapy reduces the occurrence of cardiovascular events in patients with heart failure^[11,12]. Further studies are needed to demonstrate that reduction in this marker before esophageal surgery is paralleled to a consequent reduction in the risk of AF occurrence. Our study has some limitations, such as a small number of patients, NT-proBNP determined only once after esophagectomy.

In conclusion, an elevated level of perioperative NT-

Table 2 Multivariable analysis for assessing predictors of postoperative atrial fibrillation

Variables	Odds ratio	<i>P</i>	95% CI
NT-proBNP	4.711	0.008	1.212-7.644
Postoperative hypoxia	3.111	0.027	0.0988-4.891
Thoracic-gastric dilatation	2.857	0.017	1.105-5.325
Age	2.151	0.048	0.981-4.239
History of cardiac disease	1.576	0.069	0.658-3.985
History of hypertension	1.397	0.263	0.603-2.276
Site of anastomosis	1.218	0.192	0.792-2.947
Right thorax approach	1.185	0.531	0.538-1.584
Intraoperative hypotension	1.107	0.361	0.506-2.176
Postoperative fever	1.049	0.583	0.473-2.428
History of diabetes mellitus	0.938	0.624	0.378-1.297

proBNP is a strong and independent predictor of AF occurrence in patients undergoing surgery for esophageal carcinoma.

COMMENTS

Background

Atrial fibrillation (AF) is a frequent arrhythmia after esophageal procedures and is associated with an increased morbidity and mortality. Although some risk factors for AF can be predicted after surgery for esophageal carcinoma, no accurate assessment tool or biomarker has been identified that can predict the occurrence of AF early after esophageal procedures. Therefore, a sensitive blood biomarker that can predict the occurrence of AF in patients after surgery for esophageal carcinoma with a high specificity is desirable.

Research frontiers

Previous studies have identified some risk factors as predictors of AF after surgery for esophageal carcinoma, such as postoperative hypoxia, history of obstructive pulmonary disease (COPD), thoracic-gastric dilatation, age over 65 years, male gender, and history of cardiac disease. However, the ability to accurately identify patients at a high risk for AF is still limited.

Innovations and breakthroughs

This study evaluated the role of NT-proBNP in predicting postoperative AF in patients undergoing surgery for esophageal carcinoma. The results showed that an elevated level of plasma NT-proBNP obtained before or soon after surgery for esophageal carcinoma was a strong and independent predictor of the occurrence of postoperative AF. These results may allow us to stratify patients according to their risk of postoperative AF and have important clinical implications.

Applications

An elevated level of perioperative NT-proBNP is a strong and independent predictor of AF occurrence in patients undergoing surgery for esophageal carcinoma. This finding may allow us to stratify patients with perioperative risk for AF and to plan preventive strategies for selected high-risk patients.

Terminology

N-terminal pro-brain natriuretic peptide (NT-proBNP) is a neurohormone which is stored mainly in myocytes of the cardiac ventricles and released as a result of volume and pressure overload or myocardial damage. NT-proBNP has been proved useful for diagnostic and prognostic purposes in patients with congestive heart failure and other cardiac conditions.

Peer review

In this study, the authors examined whether NT-proBNP level is associated with AF after surgery for esophageal carcinoma, showing that an elevated perioperative plasma NT-proBNP level is an independent predictor of postoperative AF in patients undergoing surgery for esophageal carcinoma. This study has certainly provided important implications for identifying higher risk patients.

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CASE REPORT

Transrectal EUS-guided FNA biopsy of a presacral chordoma-report of a case and review of the literature

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Abstract

Chordomas are rare tumors which originate from the remnants of the notochord. These tumors are locally aggressive and have a predilection for the ends of the axial skeleton. An important prerequisite for optimal management of these tumors is a correct preoperative diagnosis. The present case is the first report of the use of endoscopic ultrasound to obtain transrectal fine needle aspiration biopsy of a presacral chordoma. A review of the prior computer tomography (CT) scans allowed us to calculate the tumor volume doubling time (18.3 mo). Transrectal biopsy of chordomas is controversial, however we believe that such concerns are not justified.

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Key words: Chordoma; Endoscopic ultrasound; Spinal tumors; Trans-rectal biopsies

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INTRODUCTION

Presacral chordomas are rare tumors that pose radiographic

and clinical diagnostic challenges. In most instances, a preoperative pathological diagnosis is important in order to plan the optimal management strategy. Tumors located deep in the pelvis pose technical challenges due to the loco-regional anatomy. Although several methods of obtaining tissue [surgical, computer tomography (CT) guided and percutaneous ultrasound guided sampling] have been described, there is much controversy regarding the appropriateness of transrectal biopsy in patients with a suspected chordoma. Transrectal ultrasound-guided biopsy (TRUS) of lesions of the prostate is well established, however, the size and nonflexible nature of the transducers is a limiting factor. A Medline search using search terms chordoma, endoscopic ultrasound (EUS) (or endosonography) produced no results. In this article, we describe the first reported use of EUS with fine needle aspiration (FNA) biopsy for the characterization and diagnosis of a presacral chordoma. We discuss the pathophysiology of chordoma, and the controversies that surround the use of transmural biopsy.

CASE REPORT

A 60-year-old registered nurse who had undergone a radical prostatectomy for prostate cancer 6 years ago presented with increasing pelvic pain. The PSA was non-detectable. The patient was concerned about recurrence of the prostate cancer. A non-contrast pelvic CT scan demonstrated a presacral tumor measuring 45.9 mm by 35.3 mm (Figure 1). Pelvic CT scans from 6 years ago, that were obtained as part of his prostate cancer work-up, were reviewed. In retrospect, these showed a pubococcygeal tumor measuring 17.6 mm by 14.8 mm. Using an ellipsoid volume formula, the tumor volume doubling time was determined to be 548 d (18.3 mo).

The patient was referred for EUS, which revealed a well circumscribed, echo-rich tumor with a safe window for FNA (Figure 2). FNA biopsies were obtained using the Olympus GF-UC140P curved-linear array echoendoscope (Olympus Co., Tokyo, Japan). Quick stains suggested mesenchymal origin of the tumor. The final cytology and immunohistochemistry was diagnostic of a chordoma (Figure 3).

DISCUSSION

Chordomas are rare tumors which are locally aggressive but rarely metastasize. In the United States, the age-adjusted incidence is 0.8 per million^[1]. Chordomas are



Figure 1 Post processing sagittal reconstruction of non-contrast pelvic CT demonstrating a hypo attenuating spherical lesion anterior to the coccyx.



Figure 2 Endoscopic ultrasound with the Olympus GF-UM160 radial echoendoscope demonstrates a well circumscribed hyperechoic lesion, with some focal areas of heterogeneous echotexture, corresponding to the lesion described in Figure 1.

believed to arise from benign notochordal remnants. However, this theory has recently been challenged by Yamaguchi *et al* who proposed that chordomas instead arise from benign notochordal cell tumors^[2]. The microscopic hallmark of these tumors is the presence of characteristic large cells with numerous cytoplasmic vacuoles known as physaliphorous (Greek: droplet bearing) cells. Chordomas constitute only a small percentage of osseous or neurogenic tumors, and are rarely seen in children or adolescents. Men are affected more frequently than women^[1]. Although not malignant in the usual sense, chordomas are locally destructive and have a remarkable tendency for local recurrence after cytoreductive surgery. The tumors can also metastasize. Chordomas are rare in humans, but in a series of 533 ferrets with tumors, chordomas constituted 5% of all neoplasms and 86% of bone tumors^[3]. About one-half of chordomas are located in the sacro-coccygeal region and approximately 30%-35% are present at the base of the skull. However, chordomas can occur anywhere along the vertebral column^[1]. Macroscopically, the tumors are lobulated gelatinous and brownish-grey in color, and occasionally appear translucent. Histologically, these tumors are characterized by the presence of the above noted physaliphorous cells which are very rich in mucin and glycogen. These cells

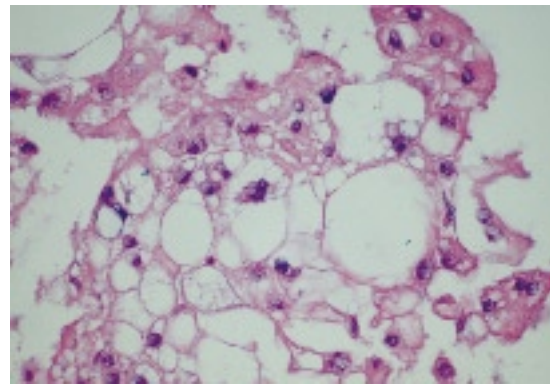


Figure 3 Tissue section of the aspirate sample shows sheets of vacuolated "physaliphorous" cells, classic of chordoma. These cells demonstrate immunoreactivity with pan-cytokeratin and epithelial membrane antigen stains.

are distributed in cords of irregular lobules embedded in a matrix of reticular fibers and an amorphous intercellular substance that contains chondroitin sulfate, hyaluronic acid and keratin sulfate^[4]. Morphologically, chordomas are similar to chondrosarcomas which, however, lack the physaliphorous cells, and some older studies without the help of immunohistochemical analysis may have inadvertently lumped these distinct entities together.

Surgical resection is the treatment of choice and radiation therapy is often used as supplemental treatment. However, since the extent of tumor resection is difficult to ascertain, and the use of terms such as local failure and disease free survival are difficult to standardize, it is difficult to interpret the results of the small series of treated patients^[5]. Recently, encouraging results have been obtained with tyrosine kinase inhibitors such as imatinib. However, a detailed discussion of the therapeutic options is beyond the scope of this report, and the interested reader is referred to a recent review published in 2007^[6].

The notion that chordomas are slow growing tumors requires qualification. As Crockard *et al* pointed out^[5], some chordomas grow fast and there appears to be a good correlation between the rate of tumor growth and prognosis. In their study of skull-base chordomas, 24 patients were followed prospectively by serial MRIs after the initial surgery to evaluate the regrowth rates. The data was correlated with histological features and Ki67 labeling index as a measure of proliferation. Volume doubling times and labeling index showed a close correlation although most of the cases clustered around volumetric doubling-time of around 25 mo. It is not clear whether growth rate of recurrent tumors is the same as the growth rate of untreated tumors, and whether growth rate of the larger sacro-coccygeal tumors is different from that of tumors at the skull-base, which become symptomatic earlier. Our patient had a volumetric doubling time of 18.3 mo which is within the expected range based on the data presented by Crockard *et al*^[5].

Controversy exists as to the best approach for biopsy, since several published reviews state that transrectal and

transmural biopsies may incur a risk of tumor tract seeding and poor outcomes. This demands a more in-depth evaluation. Needle tract seeding with tumor is rare but well described for prostate cancer. A Medline search of “chordoma-tract (or track) seeding” produced no results. The same search using the Google internet search engine retrieved as a first hit a review in a family practice journal entitled “What was this retrorectal tumor?” The hyperlink referred to the full article^[7] where a box with a red “Key Point” label stated: “A preoperative biopsy should not be performed with a retrorectal mass because of the risks of biopsy tract seeding and infection.” Two references are cited to support this advice. One of the two (a review by Fourney and Gokalan^[8]) refers to another review (Samson *et al*^[9]) as the sole supporting evidence that transrectal biopsies should not be done. We will revisit the Samson paper shortly. The other cited source^[10] is a 22 years old review which stated the following: “Of our 30 patients with chordomas, 16 received a curable resection. Nine had preoperative transrectal or trans-sacral biopsies. Recurrent tumors, including four instances of distant metastasis, subsequently developed in six of these patients. Seven patients in whom biopsy was not done received total resection, and only one patient had a recurrent tumor. In addition to recurrence, there were three complications after biopsy: two rectal abscesses and one fecal fistula. A biopsy has no role in diagnosis of retrorectal tumors unless the tumor is unresectable or highly likely to be metastatic.” Clearly, rectal biopsies or biopsies of any sort should not be done if they are followed by abscess or fistula formation in 33 percent of the cases. However, this is hardly the current experience. No causality between biopsies and poor outcomes has been established. We should also note that the authors do not discuss about the need to resect the rectum, they just observed the fact that the cases which had undergone the type of transrectal biopsy available at the time were more likely to be associated with recurrent disease in their study. No statistical tests were done. If we apply Fisher’s exact test to their data, which is appropriate here, these differences were not significant ($P = 0.0514$).

Interestingly, Fourney and Gokalan^[8] state in their review that fine needle aspiration cytology should be done in almost all cases but not through the rectum or vagina. The single supporting reference for this statement is a paper summarizing the experience with sacro-cooccygeal chordomas over a 20 years period at the Massachusetts General Hospital from 1972 to 1992 by Samson *et al*^[9]. The authors of this older series state that “in three patients the rectum and anus were included in the resection after a diverting colostomy had been performed. In two of these patients the procedure was necessary because a transrectal biopsy had been performed, which Mindell has stated to be inappropriate.” The only other information we have is that these two cases had been referred to the authors for further management. The conclusion that transrectal biopsies were the culprit was probably the opinion of the attending physician abstracted from the chart or the opinion of the reviewers. Could this be referral bias and attribution bias? Readers will note that

in one of the three cases, colostomy was necessary and no transrectal biopsy had been performed. To conclude from this limited data that transrectal biopsies lead to poor outcome appears unsound. We also do not know whether or not these biopsies were cutting (core) needle biopsies or FNA biopsies, guided by ultrasound. In the 1970’s, transrectal biopsies were often carried out with digital guidance^[11]. Mindell stated more than 25 years ago^[4]: “For instance, the biopsy should not be done through the rectum, or complete removal may necessitate resection of the rectum” but the author provided no cases or references to illustrate this belief. That the author is discussing about core biopsies rather than FNA cytology can be inferred from the following passage in the same paragraph: “Occasionally needle biopsy through a direct posterior route is satisfactory if both the surgeon and the pathologist are familiar with the techniques of obtaining adequate specimens through a needle and of preparing small plugs of tissue for histological examination”.

We believe that we have shown that there is no convincing data that transrectal biopsies of chordomas, especially if performed under real-time ultrasound guidance, are inherently more risky than transrectal biopsies of, for example, prostate cancer. There are several reports of recurrence of prostate carcinoma in the submucosa of the rectum along the needle tract as evidenced by the presence of small nodules or micrometastases observed at surgery for other reasons^[12], but the overall risk of this is extremely small. It is hard to conceive that this should be any worse in tumors which grow much more slowly than even prostate cancer. Transrectal FNA biopsies may actually have a theoretical advantage since the needle tract is typically the shortest.

CONCLUSION

We believe that biopsy of a suspected chordoma is essential. If a chordoma is found the patient should be encouraged to seek treatment at a referral center where relevant experience exists. Of all the biopsy modalities available, transrectal FNA biopsy under ultrasound guidance seems to offer the best risk-benefit ratio.

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CASE REPORT

Double aortic arch and nasogastric tubes: A fatal combination

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Abstract

Double aortic arch is a common form of complete vascular ring that encircles both the trachea and the esophagus, and presents with various respiratory and esophageal symptoms, usually in the pediatric population. We present a case of double aortic arch in an adult patient that manifested as massive upper gastrointestinal bleeding after prolonged nasogastric intubation.

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Key words: Nasogastric tubes; Aortoesophageal fistula; Gastrointestinal bleeding; Double aortic arch; Vascular rings

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INTRODUCTION

Aortoesophageal fistula is a life threatening complication and its diagnosis can often be delayed in the adult patient without any history of thoracic aortic aneurysm or esophageal malignancy. It is important to note that all imaging modalities to diagnose this entity can be unsuccessful and the best chance for patient survival is a clinical diagnosis made with confidence.

We present a case of an aortoesophageal fistula complicating nasogastric tube placement in a patient with a double aortic arch, to emphasize the importance of

clinical suspicion in diagnosing aortoesophageal fistulas, as well as the catastrophic events that might result from nasogastric tube insertion in patients with congenital aortic arch abnormalities.

CASE REPORT

A 38-year-old woman was transferred to our hospital for management of tricuspid valve endocarditis. She had a history of intravenous drug use and had been treated for 2 wk with intravenous antibiotics, but eventually required valvular replacement. On postoperative day 11, she developed massive upper gastrointestinal bleeding. Urgent endoscopy showed a large esophageal ulcer with a probable visible vessel at 22 cm from the gums. Endoscopic therapy with epinephrine injections was performed. Two hours later, she bled again, now more severely, with secondary shock. Given the location of the esophageal ulcer and the degree of bleeding, a clinical diagnosis of aortoesophageal fistula was made, and the patient was taken to the operating room, in which an aortic endograft was performed, with control of the bleeding. Complete aortoesophageal fistula repair was planned once the patient's condition stabilized. She was discharged to a subacute rehabilitation facility a few weeks later. On review of her records, it was noted that the patient had a double aortic arch that completely encased her trachea and esophagus, seen on previous chest computed tomography. The patient had a nasogastric tube for more than 5 d postoperatively for feeding and medication administration.

DISCUSSION

Double aortic arch is a complete vascular ring that encircles both the trachea and the esophagus. The most common presenting symptoms, usually in the pediatric population, are respiratory (stridor) and gastrointestinal (dysphagia)^[1-3]. The incidence of aortoesophageal fistula development in adult patients with congenital aortic arch abnormalities and prolonged nasogastric intubation has been previously reported. An extensive literature review showed an abundance of cases of congenital aortic arch abnormalities, including double aortic arch, and aortoesophageal fistula in the setting of prolonged nasogastric intubation in the pediatric population^[4-11], but only a few in the adult patient population^[12-18] (Tables 1 and 2).

The pathogenesis of this life-threatening complication is probably related to the continuous and pulsatile pressure between the aorta and the esophagus. In this anomaly, the

Table 1 Literature review of pediatric cases with congenital aortic arch abnormalities and aortoesophageal fistula after prolonged nasogastric tube placement^[10]

Author	Patient number	Age at admission	Day of upper gastrointestinal bleeding	Days of nasogastric intubation	Result	Reference
Chaikipinyo		2 mo	14th d of hospitalization	56	Lived	5
Miller		11 yr	17th d postoperatively	17	Lived	11
Woerkum		9 wk	22nd d postoperatively	43	Lived	10
Yahagi		9 d	8th d postoperatively	> 8	Lived	8
Heck	1	6 wk	14th d postoperatively	25	Died	4
	2	3 wk	7th wk of hospitalization	28	Lived	
McKeating		3 mo	17th d of hospitalization	17	Died	9
Sigalet	1	3.5 mo	59th d postoperatively	59	Died	7
	2	3 mo			Lived	
Othersen	1	5 wk	10th d postoperatively	?	Lived	6
	2	2 mo	48th d of hospitalization	48	Died	

Table 2 Literature review of adult cases with congenital aortic arch abnormalities and aortoesophageal fistula after prolonged nasogastric tube placement

Author	Age of patient (yr)	Congenital anomaly	Days of nasogastric intubation	Result	Reference
Minyard	39	Right-sided aortic arch/ RESCA	6	Died	12
Feugier	24	RESCA	?	Lived	13
Edwards	36	RESCA	?	Died	14
Merchant	17	RESCA	9	Died	15
Livesay	25	RESCA	13	Died	16
Belkin	27	RESCA	60	Died	17
Gossot	72	RESCA	30	Died	18
Massaad	38	Double aortic arch	5	Lived	

RESCA: Retroesophageal subclavian artery.

trachea and the esophagus are tightly constricted within a double aortic arch and any inserted tubes (esophageal or endotracheal) can produce pressure necrosis and a resultant fistula^[5]. Aortoesophageal fistula is a life-threatening complication and its diagnosis can often be delayed in adults without any history of thoracic aortic aneurysm or esophageal malignancy. It is important to note that all imaging modalities to diagnose this entity can be unsuccessful, and the best chance for patient survival is a clinical diagnosis made with confidence. We present this case to alert clinicians to another potential and life-threatening complication of prolonged nasogastric intubation in this specific patient population.

In conclusion, aortoesophageal fistula is a highly fatal but potentially avoidable complication in patients with vascular rings. The risks of prolonged nasogastric intubation in this patient population definitely outweigh the benefits. The diagnosis of vascular rings can often be missed in the pediatric population, and it is only when a fatal complication such as aortoesophageal fistula develops in adults that clinicians are alerted to the significance of this anomaly. The need to screen patients who are expected to have prolonged nasogastric intubation for any congenital aortic arch abnormalities should at least be suggested, if not emphasized, because the development of

an aortoesophageal fistula is a fatal complication that can be avoided with a more meticulous screening technique.

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An uncommon cause of gastro-duodenal ulceration

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Abstract

Gastrointestinal ulcers occur frequently and are mainly caused by *H pylori* infection. In this report, we present a rare case of gastro-duodenal ulcer following selective internal radiation therapy (SIRT). SIRT is a palliative treatment for unresectable liver tumours. During SIRT, ⁹⁰Y-microspheres are infused into the hepatic artery. Pre-treatment evaluation for the presence of arterial shunts to neighbouring organs should be determined in order to avoid complications of SIRT.

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Key words: Selective internal radiation therapy; Duodenal ulcer; Colon carcinoma; Hepatic metastases; Gastroscopy

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INTRODUCTION

Gastric or duodenal ulceration is generally caused by damage to the mucosal barrier of the stomach or duodenum secondary to preponderance of acid valences. In the vast majority of cases, gastric ulceration is an *H pylori*-related disease, especially in the case of duodenal ulceration. Other

causes of gastric or duodenal mucosal damage include excessive use of non-steroidal anti-inflammatory drugs, posttraumatic ischemic mucosal injury, hyperacidity caused by abuse of nicotine or changes in electrolytes, i.e. elevated serum calcium levels^[1,2].

In this report, we present a case of radiation-induced gastro-duodenal ulceration after selective internal radiation therapy (SIRT) for the treatment of hepatic metastases from a sigmoid adenocarcinoma.

Patients with hepatic metastases from a colorectal primary often die from complications associated with the impairment of liver function. Thus, in recent years development of new methods of treatment of non-resectable hepatic tumours has received much attention. External radiation is regarded as ineffective in the treatment of hepatic primary or secondary tumours since the dose of radiation that can be applied to the tumour is limited by the tolerance level of the nontumorous liver tissue^[3-5].

Intra-arterial administration of ⁹⁰Yttrium-microspheres, i.e. selective internal radiation therapy, is a palliative treatment for unresectable liver tumours such as hepatocellular carcinoma or liver metastases. This technique allows the application of high radiation dose to hepatic tumours while sparing, for the most part, normal liver parenchyma. During SIRT, ⁹⁰Y-microspheres are infused into the hepatic artery either by a surgically placed subcutaneous port or through a percutaneous femoral catheter^[3-6]. The radiopharmaceutical agent consists of non-biodegradable ⁹⁰Y-imprinted microspheres with a glass or a resin matrix with a diameter of 29-35 µm. After injection into the hepatic artery, the microspheres induce microembolization of the hepatic arterioles, mainly in the tumour tissue since its blood supply depends largely on the hepatic artery. ⁹⁰Yttrium is a beta-emitter with an average tissue penetration depth of approximately 2.4 mm. Thus, apart from microembolization, a radiation dose from 30 to 60 Gy can be applied to the tumour tissue. Furthermore radiation therapy may be followed by the selective infusion of chemotherapeutic agents into the hepatic artery^[4,7,8].

CASE REPORT

Our patient had sigmoid adenocarcinoma with disseminated hepatic metastases, which responded poorly to systemic chemotherapy with 5-fluorouracil, folinate, irinotecan and bevacizumab. SIRT was applied through a percutaneously placed femoral artery catheter. Several days after the administration of ⁹⁰Y-microspheres, the patient developed typical symptoms of upper gastrointestinal ulceration. The patient complained of epigastric pain, nausea and anorexia,

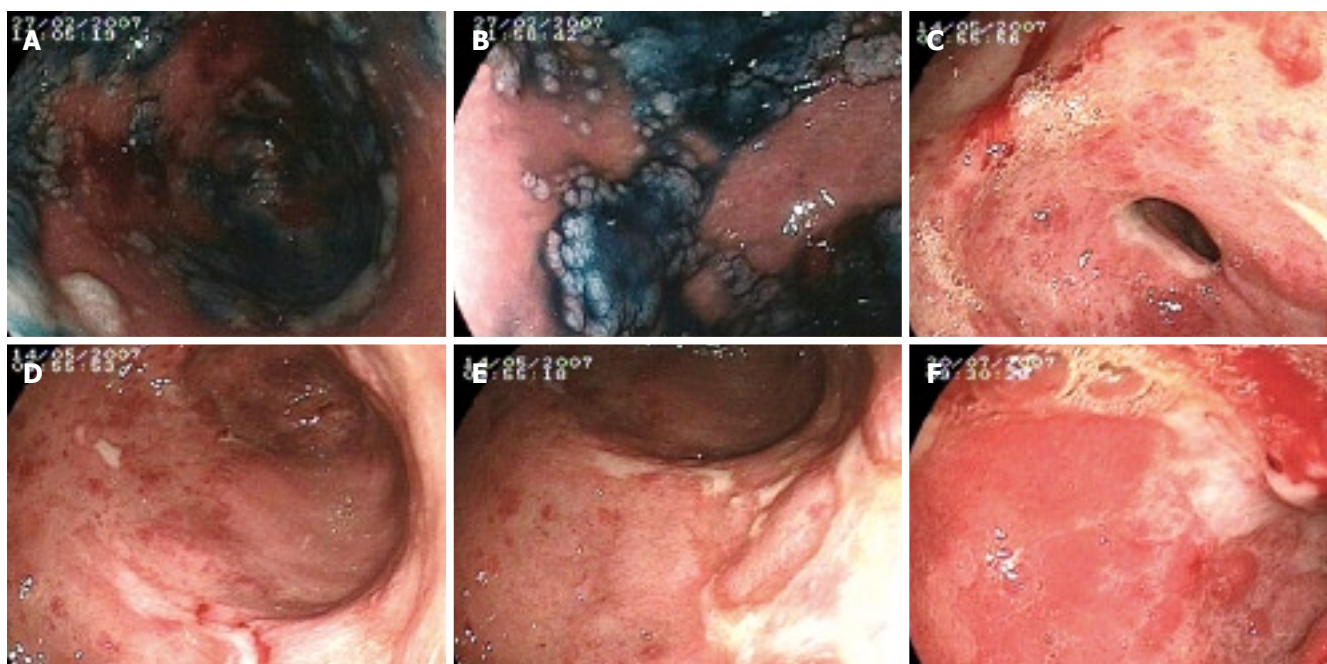


Figure 1 Chromoendoscopy. **A-B:** Chromoendoscopy with 0.6% Kongored stain showing initial mucosal damage of the gastric antrum; **C-E:** Multiple ulcerations (Forrest III) and severe mucosal inflammation of the gastric antrum, 3 mo after SIRT; **F:** Partially reepithelialized ulcer (Forrest III) in the duodenal bulb, 5 mo after SIRT.

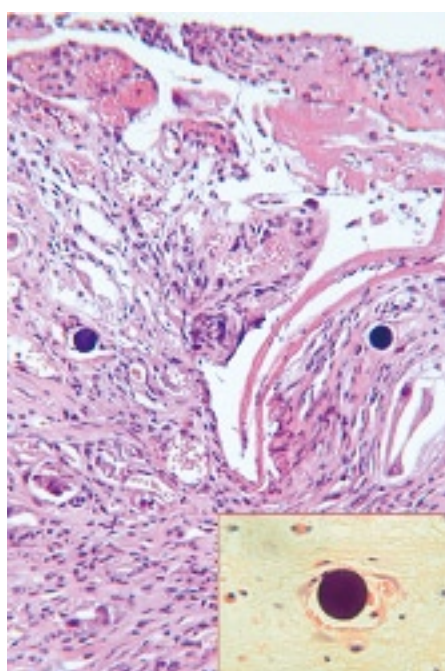


Figure 2 Gastric biopsy showing ulceration and typical inflammatory eschar at the surface. The mucosa contains granulation tissue with inflammatory cells and several small blood vessels. Two round microspheres can be seen on the right and left side of the figure. Inset: ^{90}Y -microsphere within a capillary adjacent to an erythrocyte.

followed by increasing weight loss, requiring parenteral nutrition.

Initial gastroscopy showed an extensive pangastritis with mucosal damage and a Forrest type III ulcer in the antrum and proximal duodenum which was confirmed histologically (Figures 1A, B and 2). No *H pylori*-colonization was found in gastric biopsies.

Based on these findings, the patient was treated with high dose proton-pump inhibitor therapy and sucralfate. However, the symptoms persisted and the patient experienced a further weight loss of 7 kg. Two weeks and 3 mo after the initial diagnosis, further gastroscopic examinations were performed and showed progression of the duodenal ulcer with multiple smaller Forrest III ulcers in the gastric antrum (Figure 1C-E). The ulcerations persisted even after 5 mo (Figure 1F).

Because of the close chronological association between the application of SIRT and the development of the patient's symptoms, treatment-related complication was suspected. Pathological examination was expanded to ascertain whether mucosal histological abnormalities may show typical features of radiation damage. Therefore a more detailed histological examination was conducted in order to search for microspheres in the biopsy specimens. Indeed, ^{90}Y -microspheres embolized into the capillary system were detected and photo-documented as shown in Figure 2.

Clinically, the radiation-induced ulcer persisted despite the continuous use of antacid therapy.

DISCUSSION

In general, SIRT is a well tolerated technique employed in the treatment of unresectable liver tumours, especially colorectal liver metastases. Nevertheless, complications are seen in about 20% of patients. These include radiation hepatitis and cholecystitis. A Medline literature research using terms as "gastric ulcer", "duodenal ulcer", "internal radiation" and "radiation therapy" revealed that gastroduodenal ulceration occurs in up to 12% patients after treatment with ^{90}Y -microspheres^[7-12]. Gastroduodenal ulceration has also been reported after conventional TACE with an incidence of 3% to 5.3%^[13]. However, the present

report indicates that radiogenic ulceration and radiation-induced side effects persist for a long time and are refractory to pharmaceutical therapy. Radiogenic ulceration led to significant symptoms associated with a sustained decline in the quality of life and an enduring influence on the nutritional status of the patient.

There are several options for pre-treatment planning before the use of SIRT including CT- and PET-scans, visceral angiography and the application of 99m-Techne-tium macroaggregated albumin to assess tumour vasculature, tumour volume and extrahepatic shunting^[9,14-16]. With regard to the frequently occurring side-effects of SIRT, the importance of pre-treatment assessment and pre-therapeutic embolization of arterial shunts to neighbouring organs must be established in order to avoid inappropriate loss of quality of life in these patients.

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CASE REPORT

Concomitant autoimmune and genetic pancreatitis leads to severe inflammatory conditions

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Abstract

Chronic pancreatitis characterized by an early onset should be extensively investigated including the search for a mutation of the *PRSS1*, *SPINK-1* or *CFTR* genes and potential features of autoimmune pancreatitis. We here describe a case of chronic pancreatitis with an onset at a very young age in which a mutation of the *PRSS1* and several features of autoimmune pancreatitis were identified.

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Key words: Chronic pancreatitis; Genetics; Autoimmune pancreatitis; *SPINK-1*; *CFTR*; *PRSS1*

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INTRODUCTION

Chronic pancreatitis is a progressive inflammatory disease of the pancreatic gland that will finally result in both exocrine and endocrine insufficiency^[1]. The insult to the gland is usually caused by recurrent episodes of pancreatic necro-inflammation, leading to pancreatic atrophy and dysfunction. It manifests clinically as maldigestion,

weight loss, constant or intermittent abdominal pain and eventually diabetes. Over the past decade, tremendous advance has been made regarding the etiology of chronic pancreatitis^[2]. Alcohol abuse remains the most frequent cause of chronic pancreatitis, while other causes have been delineated such as tobacco abuse, genetically determined pancreatitis and autoimmune pancreatitis^[3]. Additional causes such as pancreas divisum are controversial. Cases of recurrent acute pancreatitis and some forms of chronic pancreatitis have been definitively associated with genetic mutations^[4]. Indeed, genetic analyses have identified a specific gene for hereditary pancreatitis on the long arm of the chromosome 7 (cationic trypsinogen gene or *PRSS1*). Some *PRSS1* mutations enhance trypsinogen autoactivation, explaining the onset at young age, recurrent acute episodes of pancreatitis and progress into chronic pancreatitis. Other mutations may render some patients more susceptible to pancreatitis in the presence of other insults to the pancreas as shown in AIDS patients receiving potentially harmful drugs for the pancreas^[5]. Thus, serine protease inhibitor Kazal type 1 (*SPINK1*) and cystic fibrosis transmembrane conductance regulator (*CFTR*) genes have been involved in idiopathic recurrent acute pancreatitis and chronic pancreatitis. Since 1965, several authors have paid attention to particular cases of pancreatitis of an autoimmune pattern and some of the cases were associated with Sjögren's syndrome or primary biliary cirrhosis^[6]. This entity was recognized as autoimmune pancreatitis, a condition responsible for both acute and chronic pancreatitis.

We herein describe a case of chronic pancreatitis with an onset at a very young age, in which a mutation of the *PRSS1* and several features of autoimmune pancreatitis were identified.

CASE REPORT

A 33-year-old man approached to our pancreatic outpatient unit for information because he read on internet about recent advance made in the understanding of the causes of pancreatitis. His disease history started 23 years ago at the age of 10 years with the first episode of acute pancreatitis that required a 21-d hospital stay. The first episode was characterized by an acute onset of abdominal pain localized in the epigastric area accompanied by intractable vomiting associated with increased serum amylase concentrations twice the upper normal values. White blood cell counts were 10350/mm³ with 19% of

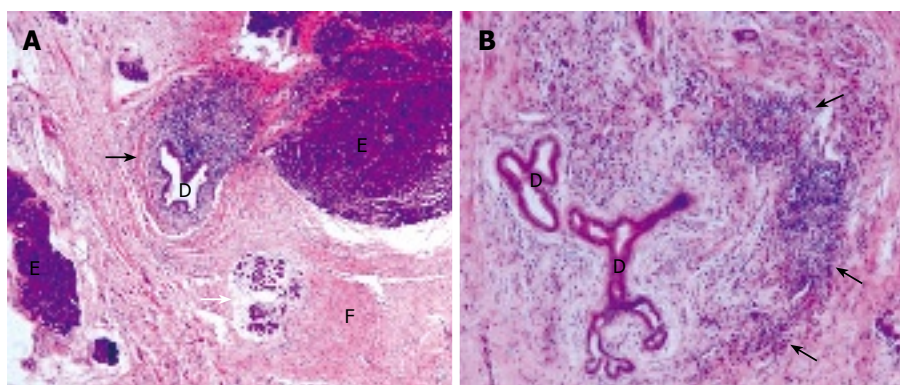


Figure 1 A: Pancreatic histological section showing the periductular infiltrate composed of lymphoplasmocytic inflammatory cells (black arrow), and areas of acinar cell necrosis (white arrow); B: High power field magnification section depicting the lymphoplasmocytic infiltrate around pancreatic ducts (black arrows), a typical feature of autoimmune pancreatitis. D: Pancreatic duct; E: Acinar cells and; F: Area of diffuse fibrosis.

band form granulocytes. Calcium, triglyceride levels and liver enzymes were within normal range. Mumps serology confirmed a previous disease. Percutaneous abdominal sonography identified an enlarged gland while the gallbladder was normal without stone. There was no familial history of pancreatic disorders and the patient had no other relevant past medical history. A conservative approach was applied while the extensive search for the etiology of acute pancreatitis remained unsuccessful after exclusion of biliary disease, alcohol abuse, drug-induced acute pancreatitis. Between 1986 and 1990, the patient subsequently developed four recurrent episodes of acute pancreatitis, the cause remains inconclusive. A sweat test was normal excluding cystic fibrosis, while there was a suspicion of pancreas divisum during endoscopic retrograde cholangiopancreatography. In 1990, the patient underwent cholecystectomy and surgical papillotomy. Transcystic cholangiopancreatography showed a normal common bile duct and a normal pancreatic ductular tract, ruling out the previous suspicion of pancreas divisum. The progress between 1990 and 1996 was marked by 5 additional episodes of pancreatitis with calcifications localized in the head and corpus of the pancreas. In 1996, a magnetic resonance imaging (MRI) performed during the 10th episode of pancreatitis identified a ductular stenosis of the caudal part of the main duct associated with distal dilatation of side branch ducts. A caudal pancreatectomy was performed without complications. Histological analysis showed pancreatic atrophy associated with large fibrous strains. Since 1996, the patient has suffered from 9 additional uncomplicated episodes of acute pancreatitis. He never drank alcohol neither was addicted to tobacco use. He was referred to us after the last severe acute pancreatitis in May 2007.

Based on the clinical presentation of the patient, we choose to investigate point mutations in the cationic trypsinogen gene (*PRSS1*) that underlie hereditary pancreatitis, although we had no data suggestive of familial pancreatitis in this patient. For *CFTR* mutations and *SPINK1* polymorphisms, we only analysed the most frequent 33 mutations associated with a more severe clinical importance. Whereas genetic testing revealed normal *CFTR* and *SPINK1* genes, *PRSS1* analysis revealed the mutation p.A16V which represents the third most common mutation in *PRSS1* besides *R122H* and *N29I*. Serum auto-antibodies including antilactoferrin antibodies, anticarbonic anhydrase antibodies type I and II were strongly positive,

whereas serum gammaglobulin levels IgG4 reached 12.8% (normal range: 2.5%-5.9%). A second interpretation of the pancreatic specimen collected in 1996 was performed in our University Hospital and clearly showed a periductular lymphoplasmocytic infiltrate and a diffuse pancreatic fibrosis (Figure 1), and all features characterized autoimmune pancreatitis.

DISCUSSION

Personal and familial histories, clinical symptoms, laboratory tests, and histological studies when available may help identify some of the etiologies of chronic pancreatitis, but 15%-25% of the cases remain of unknown origin. Recently, autoimmune diseases and genetic mutations have been described in association with pancreatic diseases. Particularly, the *PRSS1*, *R122H* and *N29I* mutations have been found to be responsible for hereditary pancreatitis, a rare cause of pancreatitis characterized by an autosomal dominant inheritance pattern and high penetrance reaching 70%-80%^[7]. Whereas the *R122H* and *N29I* mutations enhance trypsinogen autoactivation, the *PRSS1* variant p.A16V displays another harmful mechanism by increasing the activation rate of peptide processing regulated by chymotrypsin, which finally accelerates the trypsinogen activation. Moreover, the p.A16V is found most exclusively in patients without a familial history of pancreatitis, a feature shared by our patient. Molecular test represents a very effective tool to investigate the acute and chronic pancreatitis after having ruled out more common causes of pancreatitis such as biliary stones migration, alcohol-abuse and pancreatitis-induced pancreatitis^[8]. Indeed, genetic test can be used to identify unsuspected cases of genetically transmitted disease, particularly in patients without a strong history in pedigrees. Surprisingly, the patient concomitantly presented with several biological and histological features that confirm the diagnosis of autoimmune pancreatitis. To what extent autoimmune pancreatitis has influenced the course of the disease in the patient remains speculative, but two key points should be stressed. The median age of the onset of hereditary pancreatitis is 12 years^[9], while the patient became symptomatic at age 10. The pain usually improves or resolves with time in the majority of patients while the patient still had severe attacks that need a long hospital stay. The steroids would be used to treat next attack and the clinical response will be observed. If positive,

the attributable role of autoimmune pancreatitis will be definitively delineated.

In summary, we present for the first time a patient with a chronic pancreatic disease that may result from a combination of genetic mutations and autoimmune diseases.

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Extreme gastric dilation caused by chronic lead poisoning: A case report

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Abstract

Lead is a toxic metal that affects many organ systems and functions in humans. In the majority of adults, chronic lead poisoning comes from exposures to work places and can occur in numerous work settings, such as manufacturing, lead smelting and refinement, or due to use of batteries, pigments, solder, ammunitions, paint, car radiators, cable and wires, certain cosmetics. In some countries, lead is added to petrol. We present a rare case of gastric dilation caused by long-term petrol ingestion. A 16-year-old young man was admitted to our hospital due to a 6-mo history of exhaustion, dizziness, nausea, abdominal cramps and constipation. X-ray examination revealed dilated stomach descending into the pelvis and small bowel distension. After a long clinical observation, we found that the reason for the chronic lead poisoning of the patient was due to a 3-year history of petrol ingestion. The patient spontaneously recovered and stomach returned to its normal position and size. Lead poisoning should be taken into consideration in all unexplained cases of gastric dilation.

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Key words: Lead; Petrol; Stomach; Poisoning; Gastric dilation

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INTRODUCTION

Lead is a toxic metal that affects many organ systems and functions in humans. In the majority of adults, chronic lead poisoning comes from exposures to work places and can occur in numerous work settings, such as manufacturing, lead smelting and refinement or due to use of batteries, pigments, solder, ammunitions, paint, car radiators, cable and wires, certain cosmetics^[1]. In some countries, lead is added to petrol. Inorganic lead is absorbed from the respiratory or gastrointestinal tract. Approximately 30%-40% of inhaled lead is absorbed into the blood stream^[2]. Gastrointestinal absorption varies depending on nutritional status and age. Iron is believed to impair lead uptake in the gut, while iron deficiency is associated with increased blood lead concentrations in children. Once absorbed, 99% of circulating lead is bound to erythrocytes for approximately 30-35 d (only 1% of absorbed lead is found in plasma and serum) and is dispersed into the soft tissues of liver, renal cortex, aorta, brain, lungs, spleen, teeth, and bones in the following 4-6 wk. The clinical manifestations can vary from individual to individual. Diagnosis of lead toxicity is mainly based on the elevated blood lead levels (BLL). There is a general correlation between toxic effects of lead and BLL. New data implicate that lower blood lead levels, previously considered normal, can cause cognitive dysfunction, neurobehavioral disorders, different neurological damages, hypertension and renal impairment^[3-6].

Toxicity of petrol is usually associated with inhalation of vapor leading to dysfunction of the central nervous system. Ingestion of petrol by adults is rare and happens accidentally during siphoning of petrol tanks. The incidence of petrol ingestion in children is relatively low. An accidental ingestion of petrol causes acute symptoms of gastrointestinal tract including abdominal pain, nausea, vomiting and diarrhea. We report a rare case of extreme gastric dilation caused by long-term petrol ingestion.

CASE REPORT

A previously healthy 16-year-old young man was admitted to our hospital due to a 6-mo history of exhaustion, dizziness, nausea, abdominal cramps and constipation.

He was not drug addictive. At admission to our hospital, he was pale, with slightly icteric eyes, normal heart and lungs, generalized abdomen tenderness, but no hepatosplenomegaly. Neurological examination was normal. Laboratory investigation revealed normocytic anemia, 117 g/L hemoglobin, 7 $\mu\text{mol/L}$ iron (normal 11-30 $\mu\text{mol/L}$), 26 $\mu\text{mol/L}$ bilirubin (normal 0-20 $\mu\text{mol/L}$), 21 $\mu\text{mol/L}$ unconjugated bilirubin, 41 IU/L AST (normal 0-30 IU/L), 56 IU/L ALT (normal 0-41 IU/L). Blood tests showed that white blood cells, platelets, urea, creatinine, acid uric, albumin, HBsAg, anti-HCV, anti-HIV, and urine were normal or negative. Chest radiography and abdominal ultrasound were normal. X-ray examination of the stomach revealed extreme gastric dilation (Figure 1A). The stomach descended into the pelvis. Gastroscopy showed chronic erosive gastritis. Colonoscopy was normal. After supportive therapy, the patient was discharged without explanation for gastric dilation. Control hospitalization in our hospital was four months later. During that time, he was feeling well and had no abdominal pains. Physical examination at admission was normal. Laboratory investigations displayed that hemoglobin was decreased to 111 g/L and iron to 8 $\mu\text{mol/L}$ while bilirubin was increased to 32 $\mu\text{mol/L}$ (unconjugated 30 $\mu\text{mol/L}$). Red blood cells, white blood cells, aminotransferases, urea, creatinine, acid uric and urine returned to normal. Coombs test was negative. Stool examination was negative for parasites. X-ray examination of stomach was the same as in previous hospitalization. Then we intended to do more aggressive medical examinations but the patient told that he was selling petrol on the street before he had symptoms three years ago. It was an illegal work and the patient was afraid to admit it before. During the siphoning, he often swallowed a small amount of petrol. We, therefore, determined his lead blood level (BLL) which was 30 mcg/dL (normal < 10.0 mcg/dL). Urinary excretion of aminolevulinic acid was normal. It was obvious that the patient had chronic lead poisoning but we did not treat him because his condition was good due to no longer lead exposure. We saw the patient four years later during his military service. In that period, he sometimes felt nausea and paresthesia in fingers and toes. Physical examination was normal. Laboratory analyses were normal except for an increased bilirubin value of 43 $\mu\text{mol/L}$ (unconjugated 37 $\mu\text{mol/L}$). Serum lead concentration was normal (2.66 mcg/dL). X-ray examination revealed that his stomach returned to its normal size and position (Figure 1B). Nerve conduction studies showed mixed sensory-motor polyneuropathy.

DISCUSSION

Lead is widely dispersed in the environment. In the world, the main sources of lead exposure are gasoline additives, food-can soldering, lead-based paints, ceramic glaze and drinking water systems, cosmetic and folk remedies. In literature, there are single reports on long-term lead exposure and few reports on lead poisoning from retained bullets^[7,8]. Two-year consumption of homemade wine can cause lead poisoning^[9]. Chronic lead poisoning has many symptoms and signs, including pains, numbness or tingling of the extremities, muscular weakness, headache, loss mood dis-

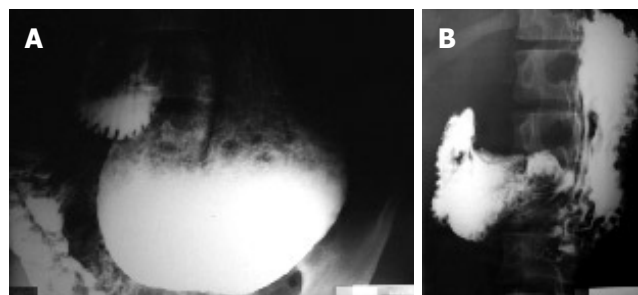


Figure 1 X-ray examination showing dilated stomach descending into the pelvis (A) and the stomach having returned to its normal size and position (B).

orders, reduced sperm count, and abnormal sperm. Our patient had mixed sensory-motor polyneuropathy and anemia. Lead has effects on cell membrane, interfering with various energy and transport systems, which may explain why it can shorten erythrocyte survival time and cause anemia. Peripheral blood smear could show basophilic stippling in several red cells from a patient with lead poisoning. Lead can interfere with the heme synthetic pathway especially enzyme delta-aminolevulinic acid dehydratase causing accumulation of aminolevulinic acid and increasing urinary excretion. Since aminolevulinic acid could be detected in urine only when BLL exceeds 35 mcg/dL, it is not a marker of low lead toxicity^[10]. Our patient had a lower BLL and normal urinary aminolevulinic acid excretion. Gastrointestinal manifestations of lead poisoning include chronic or recurrent abdominal pain, nausea, vomiting, constipation, bloating, anorexia and weight loss^[11-13]. These symptoms associated with anemia could lead to toxic etiology especially in the absence of other causes. Our patient had a 6-mo history of unexplained abdominal cramps after stopping petrol ingestion. Generally, all toxic effects of petrol are associated with vapor inhalation. A few minutes after vapor inhalation, symptoms resembling alcohol intoxication (dizziness, excitement, incoordination, *etc*) occur^[14]. Accidental ingestion of petrol is very rare and may cause symptoms of acute gastrointestinal irritation. The main health effect associated with petrol exposure is pneumonitis, resulting from pulmonary aspiration of vomitus following ingestion^[15,16]. No report on long-term petrol ingestion is available. In addition to a number of potentially neurotoxic chemicals (benzene, toluene, ethyl benzene, n-hexane, *etc*), lead in petrol is a main additive. Since 2000 in most countries, petrol has only been commercially available in unleaded form, but our patient in Serbia, was selling petrol with lead. When lead is inhaled or absorbed through skin, it is toxic to humans. We could not find any other similar case of extreme gastric dilation caused by lead. Srebocan *et al*^[17] have described gastric dilation in a dog with lead poisoning. The pathogenesis of such dilation remains uncertain, but it seems that lead has a paralytic action on gastrointestinal system. Normal gastrointestinal motor function is a complex series of events that require coordination of the sympathetic and parasympathetic nervous systems, neurons within the stomach and intestine, and the smooth muscle cells of the gut. Abnormalities of this process can delay gastric emptying (gastric stasis) and gastric tonus (gastric ptosis). It seems that reduction of heme body pool

caused by lead impairs cyto-dynamics impairing nerve conduction and paralysis of stomach. Diagnostic evaluation of adults with potential lead toxicity includes medical and environmental history, searching for potential sources of exposure, signs and symptoms of lead poisoning, and laboratory examination. BLL is essential for the diagnosis of lead poisoning. However, BLL measures current exposure to lead, but lead may also be incorporated into bone due to prior exposures not shown in BLL until this bone-lead becomes "mobilized" through pregnancy or fracture healing. X-ray fluorescence (XRF) equipment can be used to measure lead concentrations in bones^[18]. The standard elevated BLL is above 25 mcg/dL in adults and above 10 mcg/dL in children. BLL was 30 mcg/dL in our patient ten months after stopping petrol ingestion. The decision to treat it with chelating agents depends on a number of factors, including presence of lead-related symptoms, current BLL, duration of excessive lead exposure and symptoms. Patients with BLL over 80 mcg/dL should be treated^[19]. Chelation therapy is recommended for individuals with blood lead levels between 40 mcg/dL and 80 mcg/dL if they have lead-related symptoms. The main chemicals used in chelation therapy are ethylenediaminetetraacetic acid (EDTA) administered through injection and oral drug dimercaprol (BAL). Since our patient had a lower BLL, he could recover by avoiding exposure to lead.

In conclusion, this case illustrates that lead poisoning should be taken into consideration in all unexplained cases of gastric dilation.

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CASE REPORT

Systemic gemcitabine combined with intra-arterial low-dose cisplatin and 5-fluorouracil for advanced hepatocellular carcinoma: Seven cases

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Uka K, Aikata H, Takaki S, Kawaoka T, Saneto H, Miki D, Takahashi S, Toyota N, Ito K, Chayama K. Systemic gemcitabine combined with intra-arterial low-dose cisplatin and 5-fluorouracil for advanced hepatocellular carcinoma: Seven cases. *World J Gastroenterol* 2008; 14(16): 2602-2608 Available from: URL: <http://www.wjgnet.com/1007-9327/14/2602.asp> DOI: <http://dx.doi.org/10.3748/wjg.14.2602>

Abstract

The combination of intra-arterial low-dose cisplatin and 5-fluorouracil (5-FU) is effective against advanced hepatocellular carcinoma (HCC). Systemic gemcitabine chemotherapy seems effective in many cancers. We report the results of combination therapy with systemic gemcitabine, intra-arterial low-dose cisplatin and 5-FU (GEMFP). Seven patients with non-resectable advanced HCC were treated with GEMFP. One course of chemotherapy consisted of daily intra-arterial cisplatin (20 mg/body weight/hour on d 1, 10 mg/body weight per 0.5 h on d 2-5 and 8-12), followed by 5-FU (250 mg/body weight per 5 h on d 1-5 and 8-12) *via* an injection port. Gemcitabine at 1000 mg/m² was administered intravenously at 0.5 h on d 1 and 8. The objective response was 57%. The response to GEMFP was as follows: complete response (no patients), partial response (four patients), stable disease (three patients), and progressive disease (no patients). The median survival period was 8 mo (range, 5-55). With regard to the National Cancer Institute Common Toxicity Criteria (NCI-CTC) grade 3 or 4 adverse reactions, seven (100%), seven, six (86%) and one (14%) patients developed leukopenia, neutropenia, thrombocytopenia and anemia, respectively. GEMFP may potentially be effective for non-resectable advanced HCC, but it has severe hematologic toxicity.

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common neoplasms in Africa and Asia, including Japan^[1-3]. Despite advances in diagnostic techniques and therapeutic procedures [e.g. ultrasonography (US), computed tomography (CT), magnetic resonance imaging (MRI), angiography (AG), surgical resection, radiofrequency ablation (RFA), percutaneous ethanol injection (PEI), and transcatheter arterial chemoembolization (TACE)], morbidity [e.g. portal vein tumor thrombosis (PVTT) and distant metastasis], mortality rates for non-resectable advanced HCC remain poor^[4-8].

Advances in implantable drug delivery systems have made it possible to administer repeated arterial infusion of anticancer agents. Initial attempts have included monotherapy with intra-arterial 5-fluorouracil (5-FU) for non-resectable HCC^[9,10]. However, such treatment results in a low response rate (13% and 22%). Several groups have used low-dose cisplatin and 5-FU for advanced HCC with PVTT, and have reported favorable results^[11-13]. Several recent studies have reported the survival benefits of combination therapy of intra-arterial 5-FU and subcutaneous interferon alpha (IFN- α) therapy for advanced HCC with PVTT^[14-18].

Gemcitabine is a novel nucleoside analog with a broad spectrum of antitumor activity in preclinical murine leukemia and solid tumor models^[19-23]. Several studies have reported the efficiency of intravenous gemcitabine alone and in combination with other anticancer agents for advanced HCC^[21-23]. Such regimens have resulted in response rates ranging from 17.8% to 20.0%. When considered with the results of the above chemotherapy, systemic gemcitabine combined with intra-arterial chemotherapy may potentially be useful for patients with advanced HCC. However, to the best of our knowledge, there is no information about systemic gemcitabine combined with intra-arterial low-dose cisplatin and 5-FU (GEMFP) for advanced HCC. We report here the efficacy and safety of GEMFP in the treatment of seven patients with advanced HCC.

CASE REPORT

Eligibility

The treatment eligibility criteria were as follows: (1) Age 20-70 years; (2) Child-Pugh class A; (3) leukocyte count > 3500/ μ L; (4) neutrophil count > 2000/ μ L; (5) hemoglobin > 12 g/dL; (6) platelet count > 65000/ μ L; (7) total bilirubin < 2.0 mg/dL; (8) serum creatinine < 1.0 mg/dL; (9) aminotransferase < 100 IU/L (10) non-resectable HCC or not suitable for local ablation therapy because of multiple tumors or PVTT (in the first branch or trunk); (11) HCC not suitable for TACE or TACE was ineffective; (12) main tumor size > 40 mm; (13) tumor number > 5; (14) bilobular lesions; (15) Eastern Cooperative Oncology Group performance status (PS) of 0^[24]; (16) HCC without marked arteriovenous shunt; (17) HCC without marked arteriportal shunt; (18) no extra-hepatic metastases; (19) absence of other malignant diseases; (20) no recent history of upper gastrointestinal bleeding; (21) no history of heavy alcohol abuse; and (22) no other serious medical condition that would interfere with participation in this study. Participation also required signing informed consent to the study, which had been pre-approved by the Institutional Review Board of Hiroshima University.

Patients

From June 2002 to December 2006, 282 consecutive patients with non-resectable HCC were admitted to our hospital. Due to the progression of HCC (e.g. PVTT, extrahepatic metastases), these patients were not suitable candidates for either surgical resection or local ablation therapy, including RFA and PEI. Diagnosis of HCC was established based on typical hypervascular radiological features or histopathological examination of needle biopsy specimens. HCC was also assessed by US, CT and AG. Furthermore, CT was obtained during arterial portography and computerized tomographic hepatic arteriography. Further assessment of HCC was conducted by measuring α -fetoprotein (AFP) and Des- γ -carboxy prothrombin (DCP). PVTT grading based on the location of the tumor thrombus was determined according to the criteria of the Liver Cancer Study Group of Japan^[25]. Of the 282 patients with advanced HCC, seven agreed to be treated with GEMFP. Table 1 lists the baseline profiles, response,

and treatment outcomes. The seven patients were assessed retrospectively.

Treatment protocol

One course of chemotherapy lasted for 2 wk. Patients received repeated arterial infusions of anticancer agents (5-FU and cisplatin) *via* an injection port. One course of chemotherapy consisted of daily intra-arterial cisplatin (20 mg/body weight on d 1, 10 mg/body weight on d 2-5 and 8-12), followed by 5-FU (250 mg/body weight on d 1-5 and 8-12). D 6 and 7 were a rest period. Cisplatin and 5-FU were administered by a mechanical infusion pump at 20 mg/h and 250 mg/5 h, respectively. Gemcitabine at 1000 mg/m² was administered intravenously at 0.5 h on d 1 and 8. Ondansetron hydrochloride, a serotonin antagonist, was administered intravenously. Intravenous hydration was provided by saline infusion (1000 mL) to prevent nephrotoxicity during chemotherapy. In principle, GEMFP was repeated for several courses until evidence of progressive disease, worsening of PS, worsening of hepatic reserve function, unacceptable toxicity, or patient refusal to continue. A 4-8-wk rest period of no treatment was allowed after each treatment course.

Evaluation

The maximum response to treatment was assessed in all seven patients. The response was defined according to the criteria of the Response Evaluation Criteria in Solid Tumors (RECIST)^[26]. Adverse reactions were assessed every week during and after treatment using the National Cancer Institute Common Toxicity Criteria (NCI-CTC) (version 2.0)^[27].

Response and outcomes

The objective response was 57% (Table 1). No patients showed a complete response (CR) to GEMFP and four patients showed a partial response (PR). Three patients achieved stable disease (SD) (Cases 5-7). No patient showed progressive disease (PD). In the four patients with a PR (Cases 1-4), marked regression of tumor and a decrease in tumor markers were observed after the initiation of GEMFP. The median survival period was 8 mo (range, 5-55 mo). One patient was still alive (55 mo) at the end of the observation period (Case 1). Six patients died during the observation period. Three patients died of intrahepatic HCC-related liver failure (Cases 4, 6 and 7), one from respiratory failure associated with lung metastases (Case 2), one from subarachnoid hemorrhage (SAH) (Case 3), and one from aspiration-related pneumonia due to vertebra metastasis (Case 5). Each clinical course is described in detail below.

Case 1: This patient is still alive without recurrence of HCC at the end of the observation period. The patient received two courses of GEMFP and achieved PR at 1.5 mo after the initiation of GEMFP. He then underwent surgical resection for remnant HCC, and histopathological examination of the excised tumor showed HCC with massive coagulative necrosis. Six months after surgical resection, a recurrent tumor appeared in subsegment 6, with a diameter of 15 mm. The tumor was treated with RFA. No other recurrence has been detected so far.

Table 1 Baseline profiles, response, and outcome of seven patients with HCC

Case	Age (yr)	Sex	PS	Hepatitis	Child-Pugh class	AFP (ng/mL)	L3 (%)	DCP (mAU/mL)	Main tumor size (mm)	Main tumor morphology	Vascular invasion	Tumor location	Tumor volume	Previous treatment	Treatment cycles	Response	Outcome (mo)	Cause of death
1	40	M	0	HBV	A	934.3	15.9	333	50	Massive	Vp3/Vv0	Bilobular, multiple	< 50%	None	2	PR	55, alive	
2	35	M	0	HBV	A	593350	1.9	> 2000	130	Massive	Vp4/Vv3	Bilobular, multiple	≥ 50%	HAI	3	PR	10, dead	Respiratory failure due to lung metastases
3	51	M	0	HBV	A	33460	78	> 2000	80	Massive	Vp3/Vv0	Bilobular, multiple	< 50%	HAI	3	PR	8, dead	SAH due to a cerebral aneurysm
4	60	M	0	HCV	A	12.4	35.5	> 2000	45	Massive	Vp3/Vv0	Bilobular, multiple	≥ 50%	HAI	1	PR	6, dead	Intrahepatic HCC related liver failure
5	68	M	0	HCV	A	7.5	< 0.5	86	42	Nodular	Vp0/Vv0	Bilobular, multiple	< 50%	TACE	1	SD	25, dead	Aspiration-related pneumonia due to vertebra metastasis
6	64	M	0	HCV	A	127920	64.3	698	45	Nodular	Vp3/Vv0	Bilobular, multiple	< 50%	TACE	2	SD	7, dead	Intrahepatic HCC related liver failure
7	42	M	0	HBV	A	372.8	67.5	15	100	Diffuse	Vp4/Vv0	Bilobular, multiple	≥ 50%	TACE	2	SD	5, dead	Intrahepatic HCC related liver failure

Vp0: No PVTT; Vp3: PVTT in the first branch; Vp4: PVTT in the trunk; Vv0: No hepatic venous tumor thrombus; Vv3: Hepatic venous tumor thrombus in inferior vena cava; Multiple lesions; massive type HCC; Nodular, nodular type HCC; diffuse type HCC; Bilobular: Lesions; HAI: Hepatic arterial infusion chemotherapy of low-dose cisplatin and 5-FU.

Case 2: A 35-year-old man with Hepatitis B virus (HBV)-related chronic hepatitis and massive HCC (130 mm primary tumor in right hepatic lobe) and multiple intrahepatic metastases associated with PVTT in the trunk, and hepatic venous tumor thrombus in the inferior vena cava was admitted to our hospital. He was considered not suitable for surgical resection, local ablation therapy, or TACE because of the far advanced HCC. He was first treated with one course of intra-arterial low-dose cisplatin and 5-FU, which resulted in PD. Next, he received three courses of GEMFP. At 3 mo after the initiation of GEMFP, the levels of AFP and lectin-reactive AFP (AFP-L3) decreased from 593350 to 66450 ng/mL and 21 to < 0.5%, respectively. Repeat CT scans showed regression of the primary tumors, PVTT and hepatic venous tumor thrombus (Figures 1-2). Nevertheless, the patient developed severe leukopenia, neutropenia, thrombocytopenia, and anemia, which required treatment with granulocyte colony-stimulating factor (G-CSF), and platelet and blood transfusion. No bleeding tendency or infectious disease was observed. One month after the end of the three courses of GEMFP (4 mo after the initiation of GEMFP), a CT scan showed multiple lung metastases. PR was persistently seen with respect to the intrahepatic HCC. Eight months after the initiation of GEMFP, brain metastases were identified. Subsequently, the patient received systemic chemotherapy but it was ineffective. Finally, 10 mo after the initiation of GEMFP, he died of respiratory failure due to lung metastases. Regrowth of intrahepatic HCC was not observed during the follow-up period.

Case 3: This patient was treated with three courses of

GEMFP. After one course, 1 mo after the initiation of GEMFP, he achieved PR. He continued to be treated with GEMFP and remained in PR. One month after completion of three courses of GEMFP, 8 mo after the initiation of GEMFP, he died of SAH due to rupture of a cerebral aneurysm. Because bleeding tendency and hematologic toxicity were not observed at the onset of SAH, the relationship between cause of death and GEMFP could not be confirmed.

Case 4: This patient received one course of GEMFP. One month after the initiation of GEMFP, he achieved PR. He required a long (2 mo) rest period from GEMFP because of severe leukopenia, neutropenia and thrombocytopenia. During that period, regrowth of HCC appeared on CT scan. Hepatic reserve function rapidly deteriorated because of the extent of the tumor. No further chemotherapy could be used because of poor hepatic reserve function. Six months after the initiation of GEMFP, he died of intrahepatic HCC-related liver failure.

Case 5: This patient was treated with one course of GEMFP. He subsequently achieved SD, 1 mo after the initiation of GEMFP. The treatment protocol was modified (intra-arterial low-dose cisplatin and 5-FU) after the single course of GEMFP because of severe thrombocytopenia. He continued to be treated for HCC, and 14 mo after initiation of GEMFP, vertebral metastasis was noticed. The patient developed complete spinal cord injury due to vertebral metastasis, with gradual worsening of PS. Finally, PS changed to 4 and he died of aspiration-related pneumonia. He survived for 25 mo after the initiation of GEMFP.

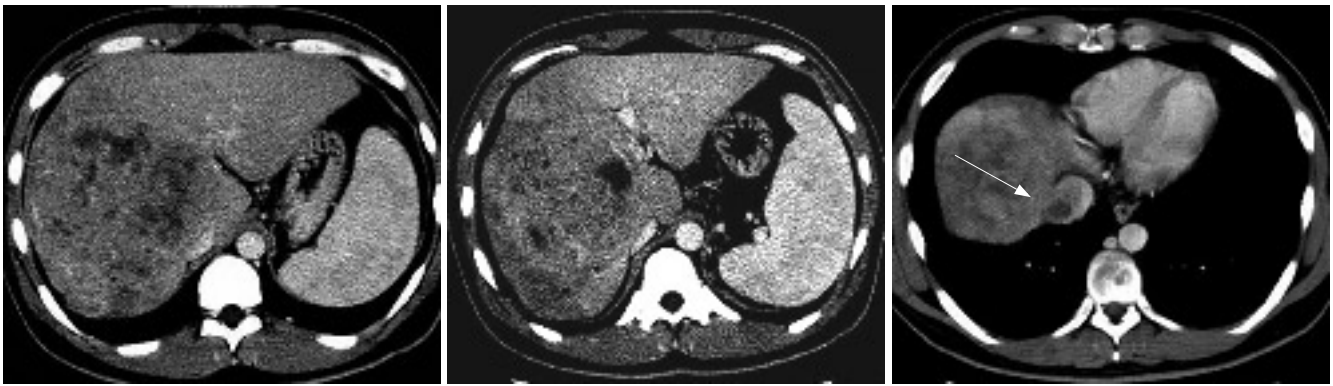


Figure 1 A CT scan showing massive HCC with PVTT in the trunk and hepatic venous tumor thrombus in the inferior vena cava (arrow). Primary tumor diameter was 130 mm in the right hepatic lobe. No portal blood flow was observed in the right first branch to the trunk.

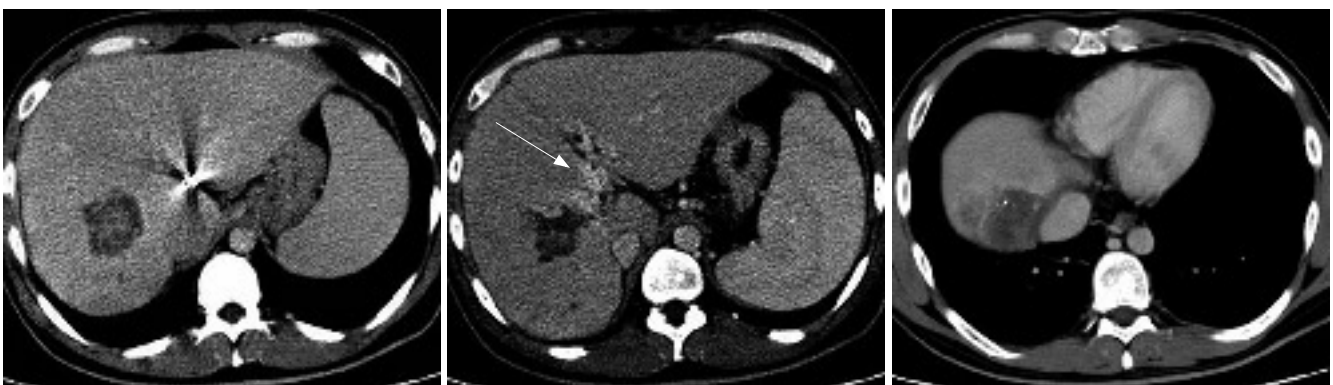


Figure 2 After three courses of GEMFP, CT scanning showed marked regression of the primary tumor (55 mm) with demonstrable portal blood flow (arrow) and the disappearance of hepatic venous tumor thrombus in the inferior vena cava.

Case 6: This patient received two courses of GEMFP and achieved SD after one course, 1 mo after the initiation of GEMFP. He continued to receive GEMFP. After 2 courses of GEMFP, 5 mo after initiation of GEMFP, CT scan showed progression of HCC and laboratory tests showed associated worsening of hepatic reserve function. Six month after the initiation of GEMFP, spontaneous rupture of HCC occurred suddenly. This resulted in progressive hepatic reserve dysfunction. Finally, he died of intrahepatic HCC-related liver failure 7 mo after the initiation of GEMFP.

Case 7: This patient received two courses of GEMFP, and achieved SD after 1 course, 1 mo after the initiation of GEMFP. He continued to be treated with GEMFP. After two courses of GEMFP, 4 mo after initiation of GEMFP, progression of HCC was noted on CT scan, together with associated obstructive jaundice and progressive hepatic reserve dysfunction. Finally, he died of intrahepatic HCC related liver failure 5 mo after initiation of GEMFP.

Adverse reactions

Table 2 summarizes the adverse reactions encountered during and after GEMFP treatment. No complications arising from catheter implantation and injection port were noted. Nausea, anorexia and anemia were mostly NCI-CTC grade 1 or 2 adverse reactions. With regard to NCI-

CTC grade 3 or 4 adverse reactions, seven, seven, six and one patients developed leukopenia, neutropenia, thrombocytopenia and anemia, respectively. Four patients (Cases 2, 4, 6 and 7) required administration of G-CSF. Five patients (Cases 1-4 and 7) required platelet transfusion, and one (Case 2) required both blood and platelet transfusion. No patients developed bleeding tendency, gastrointestinal bleeding, deterioration of hepatic function, renal damage or infectious disease.

DISCUSSION

The prognosis of patients with advanced HCC complicated with PVTT remains poor, particularly in those with PVTT in the first branches or the trunk. The median survival time of HCC patients with PVTT in the trunk is reported to be about 90 d with supportive care^[28]. Three recent studies have reported the efficacy and survival benefits of combination therapy of intra-arterial low-dose cisplatin and 5-FU for patients with advanced HCC^[11-13]. These studies included nine, 48 and 18 patients with advanced HCC and PVTT (in the second branch, first branch, or trunk), who showed objective response rates of 44% (4/9 patients), 48% (23/48 patients) and 33% (6/18 patients), respectively. The cumulative survival rates were 40% at 36 mo, 25% at 36 mo, and 28% at 12 mo, respectively. Furthermore, there has been a study of combination

Table 2 NCI-CTC grade of adverse reactions during and after GEMFP

Case	Leukocyte count (pre/end, / μ L)	Neutrophil count (pre/end, / μ L)	Hemoglobin (pre/end, g/dL)	Platelet count (pre/end, $\times 10^4$)	Nausea	Anorexia
1	3 (5050/1670)	3 (3232/835)	0 (14.6/11.8)	4 (7.5/1.5)	0	1
2	4 (7260/640)	4 (5520/420)	4 (15.7/6.2)	4 (14.8/1.8)	0	0
3	3 (3950/1720)	3 (2489/774)	0 (14.0/12.0)	4 (6.9/1.7)	0	0
4	3 (7950/1430)	4 (5168/352)	0 (16.1/11.7)	4 (20.7/2.0)	0	0
5	3 (3990/1700)	3 (2993/840)	0 (14.6/11.3)	3 (9.6/4.0)	1	0
6	3 (3840/1040)	4 (2380/357)	2 (15.2/8.5)	4 (15.3/0.5)	0	1
7	3 (6790/1950)	4 (3870/468)	2 (12.4/9.6)	2 (13.9/6.4)	0	0

therapy with intra-arterial high-dose 5-FU and cisplatin in 41 patients with advanced HCC^[29]. Objective response rate was 22% (9/41) and the cumulative survival rate was 47% at 12 mo.

A phase II study of intravenous gemcitabine monotherapy in 28 patients with non-resectable advanced and large HCC (> 10 cm) in 17 patients, extrahepatic metastases in nine, and PVTT in the trunk in 11, and the objective response rate was 18% (5/28 patients)^[21]. The median survival time of all patients treated with intravenous gemcitabine monotherapy was 19 wk, and 35 wk for those who achieved an objective response. The objective response rate was 18% (six patients) in another phase II study of intravenous gemcitabine plus intravenous oxaliplatin in 34 patients with non-resectable advanced HCC (10 patients with lung metastases, 12 with PVTT, and seven with a PS of 2)^[22], and their median survival time was 12 mo (range, 9-14 mo).

In the present study of GEMFP, the objective response rate was 57%. Compared with the objective response rates of the above studies of intra-arterial low-dose cisplatin and 5-FU (33%-48%), intravenous gemcitabine treatment (18%), and intravenous gemcitabine plus intravenous oxaliplatin treatment (18%), the objective response rate of GEMFP for advanced HCC seems better and more satisfactory. The objective response rate for intra-arterial 5-FU and subcutaneous IFN- α therapy for advanced HCC was reported to be 29% in our hospital^[18]. The objective response rate for GEMFP might be favorable compared to that for intra-arterial 5-FU and subcutaneous IFN- α therapy. The current study had a small sample size. So, a further larger, prospective randomized trial is worth considering for assessing combination therapy in patients with advanced HCC. Each chemotherapeutic agent (gemcitabine, 5-FU and cisplatin) has an antitumor effect. Cisplatin has a synergistic effect as a modulator of 5-FU^[30-32]. Although the mechanism is not clear, addition of gemcitabine might have a more potent antineoplastic effect compared with intra-arterial low-dose cisplatin and 5-FU alone. Gemcitabine may also have a biomodulator effect that enhances the antineoplastic activity of 5-FU. Gemcitabine and 5-FU might synergize each other's antineoplastic effects. Cases 2-4 had been treated with low-dose cisplatin and 5-FU before the present study, but all three showed PD. Then, the treatment protocol was changed to GEMFP, and all achieved a PR. Thus, addition of gemcitabine seemed to produce beneficial effects in these patients.

With regard to the adverse reactions to intra-arterial low-dose cisplatin and 5-FU, nausea, loss of appetite, pep-

tic ulcer, leukopenia, thrombocytopenia, deterioration of hepatic function, and renal damage have been reported in previous studies^[11-13]. Most of these adverse reactions were considered to be relatively mild and no patient required administration of G-CSF or blood transfusion. With regard to the adverse reactions associated with intravenous gemcitabine monotherapy, leukopenia, anemia, thrombocytopenia, nausea, vomiting, stomatitis, diarrhea, alopecia, skin rash, and fatigue have been reported^[21], although they were mostly mild in nature. With regard to NCI-CTC grade 3 or 4 adverse reactions associated with intravenous gemcitabine monotherapy, leukopenia (11%), anemia (14%), thrombocytopenia (11%) and deterioration of hepatic function (14%) have been reported. As for adverse reactions with intravenous gemcitabine plus intravenous oxaliplatin combination therapy, neutropenia, anemia, thrombocytopenia, neurotoxicity, nausea, vomiting, stomatitis, diarrhea, alopecia, and hand-foot syndrome have been reported^[22], which were mostly mild. With regard to NCI-CTC grade 3 or 4 adverse reactions associated with intravenous gemcitabine plus intravenous oxaliplatin combination therapy, neutropenia (24%), anemia (9%), thrombocytopenia (27%), and neurotoxicity (9%) have been reported. In the present study, hematologic toxicity was the most severe. With regard to NCI-CTC grade 3 or 4 adverse reactions, seven, seven, six and one patients developed leukopenia, neutropenia, thrombocytopenia and anemia, respectively. Compared with adverse reactions reported with intra-arterial low-dose cisplatin and 5-FU and systemic gemcitabine, the severe hematologic toxicity noted with GEMFP was considered to be mainly due to gemcitabine. However, hematologic toxicity was more severe than that seen with the above systemic chemotherapy using gemcitabine. The combination of intra-arterial low-dose cisplatin, 5-FU and gemcitabine might cause severe hematologic toxicity. Close monitoring of hematologic toxicity is very important with GEMFP. In our patients, Child-Pugh class was A and PS was 0. These limitations in part account for the good tolerance results. HCC patients with Child-Pugh class B or C, and PS > 1 might discontinue this treatment protocol.

The median survival period was 8 mo (range, 5-55 mo) in this study. The median survival of intra-arterial 5-FU and subcutaneous IFN- α therapy for advanced HCC has been reported to be 9 mo in our hospital^[18]. Four of the seven patients achieved PR. With regard to three of the four patients with PR, intrahepatic HCC was well controlled during the observation period. However, extrahepatic metastases occurred in one of these patients, and he died of respiratory failure due to lung metastases (Case 2).

Although intrahepatic HCC was well controlled by GEM-FP, GEMFP was ineffective for extrahepatic metastases. GEMFP in this study seemed to show a poor effect on extrahepatic metastases. However, this study had a small sample size. So, further, larger studies are needed to assess GEMFP. Effective treatment against extrahepatic metastases is needed. Intrahepatic HCC was not well controlled in another patient with PR (Case 4). He died of intrahepatic HCC-related liver failure, 6 mo after the initiation of GEMFP. This survival period was unsatisfactory. This was probably mainly due to the long rest period from GEMFP therapy because of severe hematologic toxicity, during which regrowth of HCC occurred. Tolerability is as important as survival. Modification of the treatment protocol might be considered to avoid hematologic toxicity.

In conclusion, we reported seven cases in which GEM-FP may have been an important component of the basic therapeutic regimen for non-resectable advanced HCC with Child-Pugh class A. Although bleeding tendency or infectious disease did not happen, hematologic toxicity was severe in our study. G-CSF and/or platelet transfusion were frequently required. A modified treatment protocol (e.g. dose reduction of gemcitabine) or inclusion criteria (e.g. leukocyte count > 5000/ μ L, neutrophil count > 3000/ μ L, hemoglobin > 12 g/dL and platelet count > 100 000/ μ L) or supportive treatment protocol using G-CSF and/or blood transfusion should be examined to avoid hematologic toxicity. Further studies are needed, including long-term follow-up, cost-benefit, and larger sample size to assess GEMFP-based chemotherapy.

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Endoscopic enucleation of gastrointestinal stromal tumors of the stomach: Report of five cases

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Abstract

Gastrointestinal stromal tumor (GIST) of the stomach was treated by endoscopic enucleation in five patients. They were three men and two woman, aged 36-56 years. Tumors located in the cardia were completely enucleated endoscopically without any serious complication. The largest diameter of removed tumors ranged from 1.2 to 2.5 cm. Histopathological diagnosis was GIST with low risk of malignancy (mitotic index < 5/50 high power field) in all cases. The patients were disease-free for 10.5-42.2 mo after endoscopic enucleation.

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Key words: Gastrointestinal stromal tumor; Endoscopic enucleation; Stomach

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INTRODUCTION

Gastrointestinal stromal tumors (GISTs) are the most common mesenchymal subepithelial tumors of the gastrointestinal tract. Immunohistochemical studies have indicated their relationship to the interstitial cells of Cajal, with which they share differentiation markers such as CD-117 (c-kit) and CD34^[1]. They are tumors with uncertain malignant potential, the risk of which is predicted by tumor size and mitotic index^[1].

Large GISTs of the stomach carry a high risk of

malignancy, and surgical resection is the gold standard for treatment of these tumors. On the other hand, small GISTs (< 3 cm in diameter) are usually benign and there is no consensus about treatment recommendations for such tumors; surgery or observation with regular follow-up^[2]. In several recent studies, laparoscopic^[3,4] or endoscopic resection^[2,5,6] has emerged as minimally invasive treatment for small GISTs of the stomach. However, most of these studies have been in single or a small number of cases, and the safety and efficacy of these novel treatments has not been established.

In this brief communication, we report the results of endoscopic enucleation of GISTs of the stomach in five patients. Endoscopic enucleation appears to be a safe and effective method of treatment of small GISTs of the stomach, if the tumor tissue does not adhere to the muscularis propria.

CASE REPORT

The patients were three men and two women, aged 36-56 years (median, 50 years). None of them complained of abdominal symptoms. Submucosal tumor of the stomach was found by endoscopy performed as a part of a medical check-up. The tumors were located in the stomach fundus in all cases, and their diameter ranged from 1.1 to 2.5 cm (median, 1.8 cm). They were diagnosed as GIST from the endoscopic appearance shown in Figure 1. Ultrasonography showed the origin of the tumor was in the submucosa, leaving the muscularis propria free from the tumor (Figure 2). Endoscopic enucleation was indicated and written informed consent to the procedure was obtained from all the patients.

Endoscopic enucleation was carried out as follows: 5-10 ml physiological saline was injected into the submucosa, and the mucosa covering the tumor was removed using a snare or a cutting knife. Then the tumor was dissected from the surrounding tissues (Figure 3A). The tumor was removed completely by snaring its base (Figure 3B). The enucleated lesion was shown in Figure 3C.

Duration of the operation was 18-45 min. (median, 25 min) and no serious complications were encountered. Several days after endoscopic removal of the tumor, oral intake was started without any trouble. Follow-up examinations at 6-8 wk after endoscopic surgery disclosed that the ulcer at the site of the enucleation had healed completely.

Histopathology of the tumor tissue was KIT-positive



Figure 1 Endoscopic appearance of a subepithelial tumor in the cardia of the stomach.

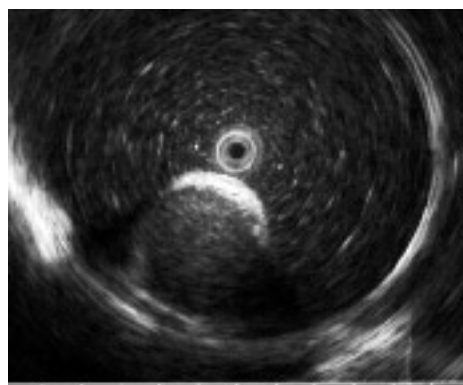


Figure 2 Ultrasonography indicated the tumor was confined within the submucosa.

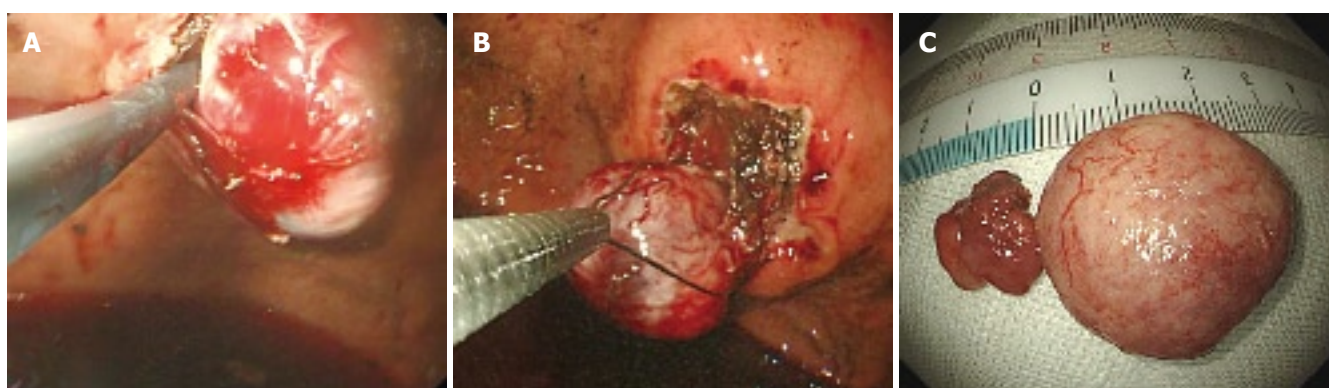


Figure 3 A: Dissection of submucosal tumor after removing the covering mucosa; B: Snaring of the base of the tumor; C: Specimens of the enucleated tumor and its covering mucosa.

GISTs with low risk for malignancy (mitotic index, < 5 per 50 high power field) in all of the patients. The patients were disease-free at 10.5-42.5 mo (median, 26.6 mo) after the operation.

DISCUSSION

Reports on endoscopic removal of small GISTs of the stomach have been increasing, but they are mostly documentation of single or a small number of cases^[2,5,6]. Park *et al*^[7] have recently reported the experience of endoscopic enucleation of submucosal tumors of the esophagus and the stomach in 15 patients. There were four patients with GISTs of the stomach. Tumor tissue was removed completely in these patients but a small perforation occurred in one patient, in whom the tumor involved the muscularis propria and had grown outward to the serosa. Roesch *et al*^[8] have treated 14 patients with submucosal tumors of the esophagus and the stomach by endoscopic surgery. In their series, there were five patients with GIST of the stomach. No serious complications were encountered in these patients but complete removal was uncertain in two patients with gastric GISTs that originated in the muscularis propria.

These reports, together with our experiences reported here, indicate endoscopic enucleation is a safe and effective method of treatment for most patients with gastric GISTs.

Endoscopic enucleation, however, is not indicated if the tumor is > 3 cm in diameter or if involvement of the muscularis propria is suspected from preoperative ultrasonography.

If GIST is completely removed by endoscopic resection and histopathological examination discloses that the risk for malignancy is low, then no further treatment is necessary. However, if resection is incomplete and/or the tumor tissue has a high risk of malignancy, additional wide resection is indicated. At present, whether postoperative imatinib can improve disease-free survival in high-risk patients is still uncertain^[9].

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CASE REPORT

Acute necrotizing pancreatitis complicated with pancreatic pseudoaneurysm of the superior mesenteric artery: A case report

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Abstract

Acute necrotizing pancreatitis complicated with pancreatic pseudoaneurysm is a rare emergency associated with high mortality that demands immediate treatment to save the patient's life. We treated a 64-year-old man who presented with a bleeding pseudoaneurysm of the superior mesenteric artery caused by acute pancreatitis, using interventional embolizing therapy. In the present report we show that interventional treatment is an effective therapeutic modality for patients with acute necrotizing pancreatitis complicated with intra-abdominal bleeding.

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Key words: Acute necrotizing pancreatitis; Bleeding; Complications; Pseudoaneurysm; Arterial embolization

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CASE REPORT

A 64-year-old man was admitted with an abrupt onset of pain in the left midsection of the abdomen. He began to feel sharp pain after dinner, which was accompanied with alcohol. The pain persisted for 8 h and became increasingly

unbearable. Blood tests showed serum amylase (AMY) of 501 IU/L, lipase (LIP) 779 IU/L and hemoglobin (Hb) 171 g/L. He was diagnosed as acute necrotizing pancreatitis based on the blood tests and findings at computer tomography (CT) scan (Figure 1A). The patient was transferred to the Department of Integrated Traditional Chinese Medicine and Western Medicine for treatment of pancreatitis, which is a special expertise of this department. The next day, the patient experienced sudden pain without any precipitating factor, accompanied with dyspnoea. Blood tests revealed that the Hb level had dropped from 171 g/L to 40 g/L. From that point on, the patient began to deteriorate rapidly and developed features of hemorrhagic shock despite continuous transfusion of 2000 mL whole blood. Severe intra-abdominal bleeding was suspected as the principal cause of his symptoms. In view of the high mortality rate of such a complication, one group of surgeons recommended prompt surgical intervention. However, after consultation with the family members we arranged an emergent angiography to identify the bleeding source. During the procedure, a ruptured pseudoaneurysm of the superior mesenteric artery (SMA) was identified, with extravasation of contrast medium from the vessel (Figure 2A and B).

Gelatin sponge pieces were infused in order to embolize the ruptured artery, and the bleeding stopped (Figure 2C). After the interventional treatment and transfusion of 400 mL of fresh blood, the Hb increased to 93 g/L. The patient was transferred to the Intensive Care Unit. Six days later, the patient was in stable condition but experienced continuous dull abdomen pain. Repeat CT scan showed that the size of the postperitoneal hematoma had increased from 6 cm to 8 cm, and a 3 cm parapancreatic cyst was noted (Figure 1A and B). The patient was operated upon to evacuate the cyst, hematoma and the necrotic tissues. A communication was made with the jejunum to create a pancreaticojejunostomy. The patient recovered gradually and was discharged from the hospital 2 wk later.

DISCUSSION

Acute pancreatitis is an inflammatory condition, which leads to acinar cell damage, interstitial edema and hemorrhage. Some patients may develop severe acute pancreatitis accompanied with multiple organ dysfunction syndrome. Acute pancreatitis is initiated by

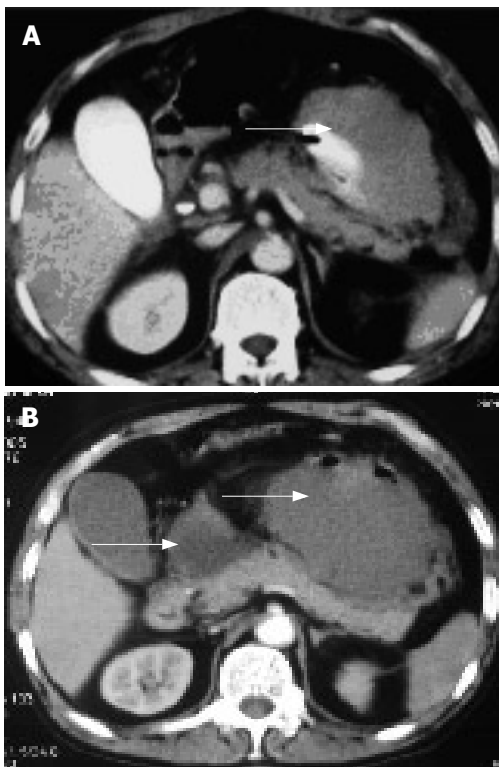


Figure 1 CT scan. **A:** Presence of the postperitoneal hematoma (arrow); **B:** Increase in the size of postperitoneal hematoma and presence of a pseudocyst (arrows).

the activation of pancreatic enzymes in the acinar cells. Local inflammation, caused by activation of trypsinogen to trypsin, is followed by the activation of inflammatory mediators. These mediators and the associated damage is not restricted to the pancreatic tissue, but may involve other organs, particularly the lungs, liver, and blood products, which are responsible for the systemic manifestations of this condition^[1].

Rupture of a pancreatic pseudoaneurysm is a rare complication of pancreatitis, and is associated with poor prognosis. The mortality correlates with the severity of the pseudoaneurysm (acute pancreatitis), and the overall mortality of this complication (severe acute pancreatitis) is about 7.8%. Angiography is usually required for confirmation of the diagnosis. Non-invasive imaging techniques such as color Doppler ultrasound and contrast-enhanced CT with sagittal or coronal reconstruction are also useful in the diagnosis of pancreatic pseudoaneurysm associated with acute necrotizing pancreatitis. Despite the advent of other imaging modalities, CT is the most effective technique to evaluate the lesion after pancreatic surgery^[2-5]. In addition, CT may demonstrate early (leakage of anastomosis, pancreatico-jejunal fistula, hemorrhage, acute pancreatitis of the remnant pancreas, peritonitis) and late (chronic fistula, abscess, aneurysms, anastomotic bilio-enteric stenosis, perianastomotic ulcers, biloma, and intra-abdominal bleeding) complications. Magnetic resonance is an alternative imaging modality when renal insufficiency or contrast sensitivity precludes the use of iodinated contrast material or when assessment of the biliary tree is the primary focus of the study. However, these imaging

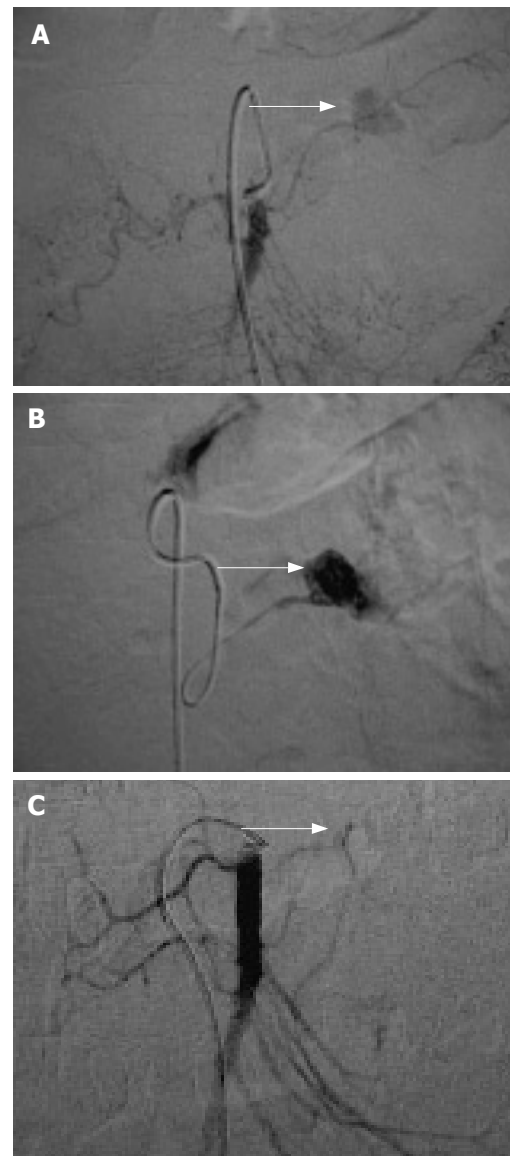


Figure 2 Angiography. **A:** SMA angiography shows a pseudoaneurysm in a branch of the SMA with extravasation of the contrast (arrow); **B:** Subselective angiography illustrates extravasation of the contrast more clearly (arrow); **C:** After embolization of the damaged artery, there is cessation of extravasation of the contrast, with disappearance of the nidus (pseudoaneurysm) (arrow).

techniques do not obviate the need for diagnostic and therapeutic angiography^[6].

With respect to therapeutic choices, endoscopic papillotomy and endoscopic removal of biliary stones is the gold standard in the treatment of gallstone-related acute pancreatitis^[6,7]. For other causes of acute pancreatitis, medical treatment should be started as early as possible with intensive care management in patients with severe acute pancreatitis. The optimal treatment is controversial. Surgical and interventional treatments, either alone or as temporizing techniques with subsequent surgery are some of the therapeutic options. However, in patients with necrotizing pancreatitis, angiographic embolization has been found to be very useful^[6,8,9], since surgical ligation or repair of the bleeding vessel is complicated by higher rebleeding rates (46%) compared to partial pancreatectomy (17%). In patients with infected pancreatic necrosis,

hemorrhage or peritonitis, surgery is the only therapeutic option, while the role of surgical treatment in the presence of sterile pancreatic necrosis accompanied with multiple organ dysfunction syndrome, which is unresponsive to medical therapy, remains unclear. Complications such as bleeding and bowel perforation are seen mostly in patients who undergo several reoperations^[10-12]. Surgery in patients with necrotizing pancreatitis carries a high mortality (about 21% for the operation^[13-15]). Surgical procedures are extremely unsafe when such patients develop acute gastrointestinal bleeding. However, angiography is the gold standard therapeutic approach in such patients. More importantly, angiography does little harm and has the highest chance of stopping bleeding in situations such as that of our patient.

Our patient was already in shock despite rapid blood transfusions when he was brought to the angio-room. As soon as we succeeded in embolizing the feeding artery, the patient's blood pressure began to improve and it was soon restored to the normal level. The subsequent treatment after embolism is as important as stopping the bleeding itself. In the present case, the patient required further surgery and intensive care treatment, resulting in gradual improvement of the patient's condition.

In summary, the present case report demonstrates that interventional treatment is an effective treatment for patients with acute necrotizing pancreatitis complicated with intra-abdominal bleeding, especially in patients who are in poor condition and those who cannot tolerate laparotomy. We wish to emphasize that stopping the bleeding is only the first step in the treatment of such patients and a close cooperation between the physicians and surgeons is required for optimal results.

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Lower gastrointestinal bleeding: Association with Sevelamer use

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Author contributions: Madan P saw the patient and wrote the first draft; Chandra P and Bhayana S revised the manuscript for important intellectual content; Hughes JI did endoscopy on the patient, provided us with pictures and revised the manuscript for important intellectual content.

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Abstract

Stercoral ulceration results from impaction of hard fecal mass on the colonic wall and is a relatively unknown cause of lower gastrointestinal bleeding. In this report, we describe a case of lower gastrointestinal bleeding due to stercoral ulceration resulting from Sevelamer, a drug which is commonly associated with constipation.

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Key words: Stercoral Ulcer; Sevelamer; Chronic renal failure; Lower gastrointestinal bleeding

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TO THE EDITOR

Stercoral ulceration is defined as an ulcer caused by pressure necrosis from hard fecal mass (fecaloma) pressing on the colonic wall. It is a relatively unknown cause of lower gastrointestinal bleeding^[1]. A prolonged history of constipation is present in almost 80% of the patients with this condition^[1]. Sevelamer, a phosphate binder, is often used in treatment of hyperphosphatemia associated with chronic

kidney diseases. It is associated with constipation in 8%-10% of the patients^[2]. To the best of our knowledge, this is the first case in which stercoral ulceration as a cause of lower gastrointestinal bleed has been ascribed to Sevelamer use.

A 62-year-old woman presented with bleeding per rectum for one day. She reported a history of constipation which occurred 1 mo ago when she started taking Sevelamer. She had not had a bowel movement for several days and when she started straining in the toilet, "something gave way" and she passed two hard stools and started bleeding from rectum. The blood was bright red in color. She felt lightheaded and was brought to the hospital. Her previous medical history was significant for diabetes mellitus, end-stage renal disease, coronary artery disease and cerebrovascular accident with no residual deficit. Besides Sevelamer, she was also taking Clopidogrel but never had similar episodes of bleeding before. Physical examination revealed tachycardia and hypotension. Her hemoglobin dropped from baseline of 12 gm/dL to 9.5 gm/dL. She was stabilized with intravenous fluid, red blood cell transfusions and subjected to colonoscopy which revealed stercoral ulcers in rectum (Figure 1). Histological examination of the ulcer showed denuded mucosa with acute and chronic inflammation. Her bleeding stopped spontaneously and she did not have any further episodes of bleeding and was later discharged on stool softeners after stopping the offending drug.

The exact prevalence of stercoral ulceration in general population is unknown. Autopsy studies have revealed presences of stercoral ulceration in about 1.3%-5.7% of elderly institutionalized patients^[3,4]. Stercoral ulcers are usually asymptomatic but may cause two dreaded complications: perforation and bleeding. However, these complications are rare in literature. Stercoral ulceration was first described in 1894 and since then less than 100 cases of stercoral ulceration associated perforation have been reported^[1]. The number of stercoral ulceration presenting as hematochezia is even lower.

Severe constipation is considered to be the main causative factor in formation of stercoral ulceration and is present in 81% of the patients^[1]. The elderly, debilitated or institutionalized patients on multiple medications are affected most frequently. Stercoral ulceration with consequent perforation has been reported in association with narcotics, amitriptyline, verapamil, immunosuppressive agents, aluminum based antacids and non-steroidal anti-inflammatory drugs (NSAIDs)^[5-8]. The effect of NSAIDs might be related to its inhibitory effect on formation of

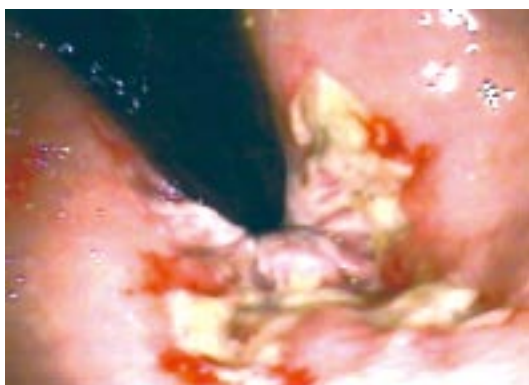


Figure 1 Stercoral ulcers in the rectum.

mucosa protecting prostaglandins rather than constipation. We could not find any report in which stercoral ulceration had been found as the consequence of Sevelamer use.

Stercoral ulcers are caused by pressure necrosis from the hard fecaloma pressing on the colonic wall. The contours of stercoral ulcers are often well-demarcated corresponding to the shape of fecolith. A fecaloma is most likely to form in the distal colon where the stools are in the most dehydrated form. The fecaloma is frequently impacted at the site of recto-sigmoid junction which is also the narrowest part of the colon. As a result of impaction, the intraluminal pressure often exceeds the capillary perfusion pressure of the bowel wall predisposing the mucosa to necrosis and ulceration^[1].

Treatment of stercoral ulceration depends upon the presenting symptoms. Complications arising from stercoral ulceration may lead to a 50% mortality in the population

at risk due to accompanying comorbidities. The stercoral perforation is usually treated surgically and those presenting with massive hemorrhage may require emergent resection or endoscopic therapy^[9].

A common clinical problem such as constipation can sometimes cause relatively unknown but potentially fatal complication such as stercoral ulceration. An internist should monitor development of constipation in a patient who is newly started on Sevelamer.

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Is the required therapeutic effect always achieved by racemic switch of proton-pump inhibitors?

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Abstract

Many of the drugs currently used in medical practice are racemates. The enantiomers of a racemic drug differ in pharmacodynamics and/or pharmacokinetics, thus in some cases it is preferable to develop pure enantiomers by racemic switch. In a recent study by Pai *et al*, dexrabeprazole [R(+)-rabeprazole] (10 mg) was found to be more effective than rabeprazole (20 mg) in the treatment of gastroesophageal reflux disease. We read with great interest in this study and discussed whether such racemic switch would be applicable to other proton-pump inhibitors (PPIs). A literature review indicates that stereoselective pharmacokinetics, rather than stereoselective pharmacological activity, is the main cause of differences in clinical efficacy between pure enantiomer and racemic PPI. Racemic switches of PPI provide the therapeutic advantages such as reducing metabolic load on the body, simplifying pharmacokinetics, providing benefit to the non-responders to standard dose of racemate, more homogenous response to treatment and better efficacy with equal safety. Further studies in quantitative structure-activity relationships (QSARs) are needed to address the fact that the preferred enantiomer of PPI is not always in the same absolute configuration, i.e., S-form is for omeprazole, pantoprazole and

tenatoprazole whereas R-form is for lansoprazole and rabeprazole.

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Key words: Proton-pump inhibitors; Enantiomer; Racemate; Stereoisomerism; Racemic switch; Pharmacokinetics; Pharmacodynamics; Cytochrome P450; Genotype

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TO THE EDITOR

We read with great interest in the study by Pai *et al*^[1], who compared the therapeutic outcomes of dexrabeprazole (10 mg) with rabeprazole (20 mg) in the treatment of gastroesophageal reflux disease (GERD). The results showed that efficacy of dexrabeprazole (10 mg) is better than rabeprazole (20 mg), with regards to improvement/healing of endoscopic lesions and relief from symptoms of regurgitation. Rabeprazole is a racemic mixture of two enantiomers, R(+)-enantiomer and S(-)-enantiomer in 1:1 proportion. Dexrabeprazole is the chirally pure R(+)-enantiomer which is more effective than the racemate and S(-)-rabeprazole in inhibiting acid-related gastric lesions in rats^[2]. A superior pharmacokinetic profile, i.e., higher maximal plasma concentrations (C_{max}) and area under the curve (AUC), was observed with R(+)-rabeprazole compared to its S(-)-enantiomer^[3]. Dexrabeprazole was launched as Dexpure[®] by Emcure Pharmaceuticals Ltd in September 2007.

Racemic switch stands for the development in single-enantiomer form of a drug that was first approved as a racemate. We have reported that the enantiomers of a racemic drug differ in pharmacodynamics and/or pharmacokinetics as a consequence of stereoselective interaction with optically active biological macromolecules, and the decision to perform racemic switch should be based on enough evidence^[4,5]. As far as proton-pump inhibitors (PPIs) are concerned, is the required therapeutic

effect achieved by racemic switch? We would discuss and share our perspectives below.

Omeprazole, lansoprazole, pantoprazole and rabeprazole possess asymmetric sulfur in their chemical structure and have been typically used in clinical practice as a racemic mixture. Esomeprazole, S(-)-enantiomer of omeprazole, is the first enantiomerically pure PPI and the compound is now marketed as Nexium[®]. The enantiomers of omeprazole produce similar pharmacological effects^[6]. The metabolic profile of S(-)-omeprazole is distinct from that of R(+)-omeprazole. Both enantiomers are metabolized by CYP2C19, but the enzyme plays a less role (73% *vs* 98%) in the metabolism of the S(-)-enantiomer. As a result, the pharmacokinetic profile of the S(-)-enantiomer is less dependent on CYP2C19 genotype, hence leading to a less interpatient variability in clearance than omeprazole. $AUC_{po(PM)}/AUC_{po(EM)}$, the ratio of *AUC* after oral administration (*AUC*_{po}) derived from poor metabolizers (PM) and extensive metabolizers (EM), is 3.0 and 7.4 for esomeprazole and omeprazole^[7], respectively. Moreover, S(-)-omeprazole is cleared more slowly and has an improved oral bioavailability (81%-98% *vs* 35%-65%), leading to the greater inhibition of gastric acid secretion compared to omeprazole.

Lansoprazole is extensively metabolized by CYP2C19 and CYP3A4 in the liver. CYP2C19 genotype influences the disposition of S(-)-lansoprazole to a greater extent than the R(+)-enantiomer, resulting in less interpatient variability in clearance with R(+)-lansoprazole compared to lansoprazole. Both enantiomers of lansoprazole possess equal potency. Therefore, the use of R(+)-lansoprazole alone would be highly desirable for clinical application^[8].

Pantoprazole is a racemic mixture of two enantiomers. Animal studies confirmed that S(-)-pantoprazole is more potent than R(+)-enantiomer in inhibiting acid-related lesions^[9]. Pantoprazole is metabolized mainly by CYP2C19 followed by sulfation and, to a lesser extent, by CYP3A4. The enantiomers of pantoprazole are differentially affected by CYP2C19 genotype. The $AUC_{po(PM)}/AUC_{po(EM)}$ ratio is 11, 2.5 and 6.0 for the R(+)-enantiomer, S(-)-enantiomer and pantoprazole, respectively^[10]. The pharmacokinetics of S-pantoprazole depend less on CYP2C19 genotype, resulting in uniform therapeutic plasma levels of the drug, thus providing benefit to the non-responders to standard dose of racemic pantoprazole. A comparative clinical trial of S(-)-pantoprazole versus racemic pantoprazole in the treatment of GERD has been carried out by Pai *et al*^[11]. S(-)-pantoprazole (20 mg) was found to be more effective than racemic pantoprazole (40 mg) in improving symptoms. S(-)-pantoprazole exhibits both pharmacokinetic and pharmacodynamic advantages and this compound known as PANPURE[®] was developed by Emcure Pharmaceuticals Ltd in 2006.

Tenatoprazole is a new PPI under clinical development by Negma-Gild. It is a racemic mixture of two enantiomers. Significant stereoselective differences in pharmacodynamics were observed in *in vivo* studies in rats and dogs, with S(-)-tenatoprazole being a eutomer^[12]. The S(-)-enantiomer is metabolized approximately 7 times more slowly than the R(+)-enantiomer, resulting in a much longer mean residence

time in the human body and an improved tissue exposure to S(-)-tenatoprazole in comparison with the R(+)-enantiomer. The S(-)-enantiomer is mainly metabolized *via* CYP3A4, which can compensate for a potential deficiency or blockade of CYP2C19. The R(+)-enantiomer is metabolized via two pathways, i.e., mainly the CYP2C19 and, to a lesser extent, by CYP3A4. Clinical studies showed the linear pharmacokinetic and pharmacodynamic characteristics of S(-)-enantiomer after 7-d treatment of tenatoprazole. In contrast, the plasma concentration of the R(+)-enantiomer is not linear, thus being not predictive of the efficacy and the tolerability of the drug. Furthermore, the pharmacokinetics of S(-)-enantiomer shows a markedly lower inter-subject variability compared to that of the R(+)-enantiomer, hence a better use of the product and a more homogenous response to treatment in all patients. Consequently, S(-)-tenatoprazole is a promising PPI with a safe general pharmacological profile.

In conclusion, stereoselective pharmacokinetics, rather than stereoselective pharmacological activity, is the main cause of differences in clinical efficacies between pure enantiomer and racemic PPI (e.g. omeprazole, lansoprazole, pantoprazole, rabeprazole and tenatoprazole). Racemic switches of PPIs provide therapeutic advantages such as reducing metabolic load on the body, simplifying pharmacokinetics, providing benefit to the non-responders to prior standard dose of racemate, more homogenous response to treatment and better efficacy with equal safety. Further studies on the quantitative structure-activity relationships (QSARs) are needed to address the fact that the preferred PPI enantiomer is not always in the same absolute configuration, i.e., S-form is for omeprazole, pantoprazole and tenatoprazole whereas R-form is for lansoprazole and rabeprazole.

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www.easl.ch/hepatitis-conference

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

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

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

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

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

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

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

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

April 23-27, Milan, Italy
 43rd Annual Meeting of the European

Association for the Study of the Liver
www.easl.ch

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

May 18-21, San Diego, California, USA
 Digestive Disease Week 2008
 June 4-7, Helsinki, Finland

The 39th Nordic Meeting of Gastroenterology
www.congrex.com/ngc2008
 June 6-8, Prague, Czech Republic
 3rd Annual European Meeting: Perspectives in Inflammatory Bowel Diseases
 Email: meetings@imedex.com

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

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

June 25-28, Lodz, Poland
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 E-mail: office@epc-iap2008.org
www.e-p-c.org
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June 26-28, Bratislava, Slovakia
 5th Central European Gastroenterology Meeting
www.ceurgem2008.cz
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11th World Congress of the International Society for Diseases of the Esophagus
 Email: isde@isde.net

September 13-16, New Delhi, India
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 Prague Hepatology Meeting 2008
www.czech-hepatology.cz/phm2008

September 20-21, Mainz, Germany
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Fat to Viruses
 September 24-27, Nantes, France
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October 18-22, Vienna, Austria
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 59th AASLD Annual Meeting and Postgraduate Course
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www.ngm2008.com

November 12, Santiago de Chile, Chile
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- 3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

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- 6 21st century heart solution may have a sting in the tail. *BMJ*

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

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

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