ISSN 1007-9327
CN 14-1219/R

World Journal of Gastroenterology®

Indexed and Abstracted in:

Current Contents®/Clinical Medicine, Science Citation Index Expanded (also known as SciSearch®) and Journal Citation Reports/Science Edition, *Index Medicus*, MEDLINE and PubMed, Chemical Abstracts, EMBASE/Excerpta Medica, Abstracts Journals, *Nature Clinical Practice Gastroenterology and Hepatology*, CAB Abstracts and Global Health.
ISI JCR 2003-2000 IF: 3.318, 2.532, 1.445 and 0.993.

Volume 14 Number 12
March 28, 2008
World J Gastroenterol
2008 March 28; 14(12): 1805-1968

Online Submissions
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www.wjgnet.com
Printed on Acid-free Paper
世界胃肠病学杂志

World Journal of
Gastroenterology ®

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2007-2009

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World Journal of Gastroenterology®

Weekly Established in October 1995

Volume 14 Number 12
March 28, 2008

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World Journal of Gastroenterology
Volume 14 **Number** 12 **March** 28, 2008

Contents

World Journal of Gastroenterology
Volume 14 **Number** 12 **March** 28, 2008

Contents

NAME OF JOURNAL
World Journal of Gastroenterology

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E-mail: wjg@wjgnet.com

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

PRINTING
Beijing Kexin Printing House

OVERSEAS DISTRIBUTOR
Beijing Bureau for Distribution of Newspapers and Journals
(Code No. 82-261)
China International Book Trading Corporation PO Box 399, Beijing, China (Code No. M4481)

PUBLICATION DATE
March 28, 2008

EDITOR-IN-CHIEF
Lian-Sheng Ma, *Taiyuan*

SUBSCRIPTION
RMB 50 Yuan for each issue, RMB 2400 Yuan for one year

CSSN
ISSN 1007-9327
CN 14-1219/R

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World J Gastroenterol 2008 March 28; 14(12): 1805-1809
World Journal of Gastroenterology ISSN 1007-9327

EDITORIAL

Intragastric injection of botulinum toxin for the treatment of obesity. Where are we?

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Received: January 4, 2008 Revised: January 29, 2008

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.

Key words: Botulinum toxin; Obesity; Gastric emptying; Gastric motility; Gastroparesis

Peer reviewers: Yvan Vandenplas, Professor, Department of Pediatrics, AZ-VUB, Laarbeeklaan 101, Brussels 1090, Belgium; Andrew Ukleja, MD, Assistant Professor, Clinical Assistant Professor of Medicine, Director of Nutrition Support Team, Director of Esophageal Motility Laboratory, Cleveland Clinic Florida, Department of Gastroenterology, 2950 Cleveland Clinic Blvd., Weston, FL 33331, United States

Garcia-Compean D, Maldonado Garza H. Intragastric injection of botulinum toxin for the treatment of obesity. Where are we? *World J Gastroenterol* 2008; 14(12): 1805-1809 Available from: URL: http://www.wjgnet.com/1007-9327/14/1805.asp DOI: http://dx.doi.org/10.3748/wjg.14.1805

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 Botulínum 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

Botulínum 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 osmolarity (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
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 *vs* saline antrum	8	Early satiety: Yes Reduction of body weight: No Gastric emptying: Negative
Foschi[27]	24	RCT[1]	200 *vs* 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 *vs* saline /antrum	24	Early satiety: No Reduction of weight: No

[1]RCT: 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 arquatte 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 (^{99}m)Tc-pertechnetate intravenously and (111) In-diethylenetriaminepentaacertic 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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S- Editor Zhong XY **L- Editor** Rippe RA **E- Editor** Lu W

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World J Gastroenterol 2008 March 28; 14(12): 1810-1811
World Journal of Gastroenterology ISSN 1007-9327

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Colorectal cancer risk in Crohn's disease

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Received: January 3, 2008 Revised: February 18, 2008

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.

Key words: Colon cancer; Crohn's disease; Surveillance; Small bowel cancer

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Freeman HJ. Colorectal cancer risk in Crohn's disease. *World J Gastroenterol* 2008; 14(12): 1810-1811 Available from: URL: http://www.wjgnet.com/1007-9327/14/1810.asp DOI: http://dx.doi.org/10.3748/wjg.14.1810

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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S- Editor Li DL **L- Editor** Alpini GD **E- Editor** Ma WH

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World J Gastroenterol 2008 March 28; 14(12): 1812-1822
World Journal of Gastroenterology ISSN 1007-9327

REVIEW

Role of chemotherapy and novel biological agents in the treatment of elderly patients with colorectal cancer

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Received: November 9, 2007 Revised: December 15, 2008

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Rosati G, Bilancia D. Role of chemotherapy and novel biological agents in the treatment of elderly patients with colorectal cancer. *World J Gastroenterol* 2008; 14(12): 1812-1822 Available from: URL: http://www.wjgnet.com/1007-9327/14/1812.asp DOI: http://dx.doi.org/10.3748/wjg.14.1812

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.

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

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	
	if can tolerate treatment	Standard cancer treatment
	if cannot tolerate treatment	Palliation
	Life expectancy not shortened by cancer	Palliation
3	Frail patients who are totally dependent with ≥ 3 comorbidities or 1 geriatric syndrome	Palliation

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 survival. 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/m^2 twice daily for 2 wk on/1 wk off) with the Mayo Clinic regimen (bolus 5-FU 425 mg/m^2 days with LV 20 mg/m^2 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/m^2 per day) plus oral LV (75 or 90 mg per day) for 28 d every 5 wk was compared with 5-FU (425 mg/m^2 per day) plus LV (20 mg/m^2 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/m^2) 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/m^2 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/m^2 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 toxicity and survival according to age during a CPT-11-based treatment of 339 patients with fluropyrimidine-resistant advanced CRC. All patients commenced CPT-11 at 350 mg/m^2 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/m^2 to 300 mg/m^2 when administered in a three-weekly schedule or from 125 mg/m^2 to 100 mg/m^2 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/m^2) 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/m^2 for oxaliplatin and 60 mg/m^2 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/m^2 for oxaliplatin on d 1, and 2000 mg/m^2 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/m^2 as an intravenous 3-h infusion on d 1 and 8 plus UFT 300 mg/m^2 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]. Massacesi *et al* examined 78 patients aged ⩾ 70 years and ECOG performance status ⩽ 2. Their schedule was: Oxaliplatin 45 mg/m^2 + 5-FU bolus 400 mg/m^2 + 5-FU infusional 600 mg/m^2 + LV 200 mg/m^2, 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/m^2 on d 1 + 5-FU bolus 300 mg/m^2, 5-FU infusional 450 mg/m^2, and LV 150 mg/m^2 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/m^2 as initial dose and 250 mg/m^2 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 $\geqslant$ 70 years could be of interest[66]. 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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World J Gastroenterol 2008 March 28; 14(12): 1823-1827
World Journal of Gastroenterology ISSN 1007-9327

REVIEW

Crosstalk between tumor cells and microenvironment *via* Wnt pathway in colorectal cancer dissemination

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Received: November 3, 2007 Revised: January 30, 2008

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.

Key words: Invasion; Microenvironment; Colorectal cancer; Epithelial-mesenchymal transition; Wnt; β-catenin

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Huang D, Du X. Crosstalk between tumor cells and microenvironment *via* Wnt pathway in colorectal cancer dissemination. *World J Gastroenterol* 2008; 14(12): 1823-1827 Available from: URL: http://www.wjgnet.com/1007-9327/14/1823.asp DOI: http://dx.doi.org/10.3748/wjg.14.1823

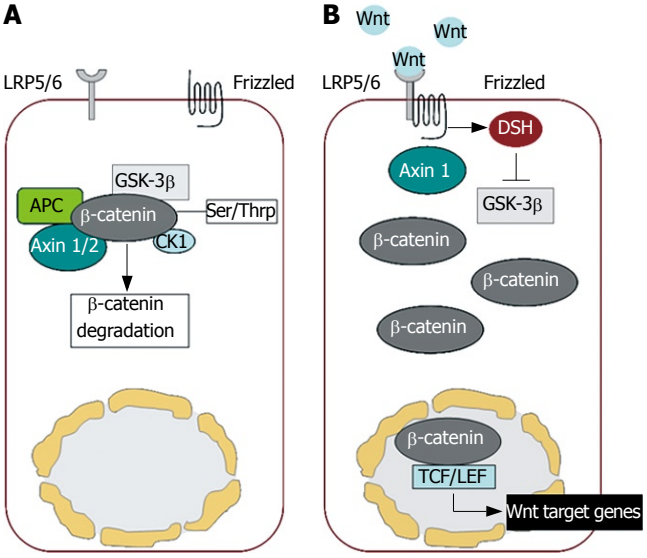
INTRODUCTION

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-



Modified from Fodde and Brabletz, *Curr Opin Cell Biol*, 19, 152, (2007)

Figure 1 Schematic illustration of the canonical Wnt/β-catenin signaling pathway. **A**: In the absence of Wnt ligands, destruction complex phosphylates 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 Dishevlled (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/Ca^{2+} 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 prerequisite. However, non-canonical pathway *via* Wnt5a is critical for macrophage-induced invasiveness[19].

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

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-medicated 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-GSK3β-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.

MICROENVIROMENTAL 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, *Foxf*1 and *Foxf*2, can limit paracrine Wnt signaling and promote extracellular matrix production in gut, and deletion of *Foxf*1 and *Foxf*2 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 GSK3β[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 (TGFβ) can also enhance Wnt/β-catenin signaling by posphorylating 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 Ake by free G protein and is directly associated with the G protein signaling (RGS) domain of axin, thus leading to GSK3β 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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S- Editor Li DL **L- Editor** Wang XL **E- Editor** Liu Y

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World J Gastroenterol 2008 March 28; 14(12): 1828-1835
World Journal of Gastroenterology ISSN 1007-9327

ESOPHAGEAL CANCER

Comparative genomic hybridization analysis of genetic aberrations associated with development of esophageal squamous cell carcinoma in Henan, China

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Received: August 17, 2007 Revised: January 6, 2008

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.

Key words: Comparative genomic hybridization; Genetic alterations; Esophageal squamous cell carcinoma; Metastatic lymph nodes

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Qin YR, Wang LD, Fan ZM, Kwong D, Guan XY. Comparative genomic hybridization analysis of genetic aberrations associated with development of esophageal squamous cell carcinoma in Henan, China. *World J Gastroenterol* 2008; 14(12): 1828-1835 Available from: URL: http://www.wjgnet.com/1007-9327/14/1828.asp DOI: http://dx.doi.org/10.3748/wjg.14.1828

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 161/100000 for male and 103/100000 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℃ 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℃. 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℃ for 5 min. The slide containing normal metaphase spreads was treated with RNase (100 mg/L) at 37℃ for 1 h and then denatured at 75℃ in 70% formamide, and 2 × SSC for 5 min. Hybridization with probes was then carried out at 37℃ in a moist chamber for 72 h. The slide was then washed in 0.4 × SSC/0.3% NP-40 at 75℃ 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 (Photometics, 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 MYCC 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 ESSC may indicate a specific tumor suppressor gene at this locus involved in ESSC 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

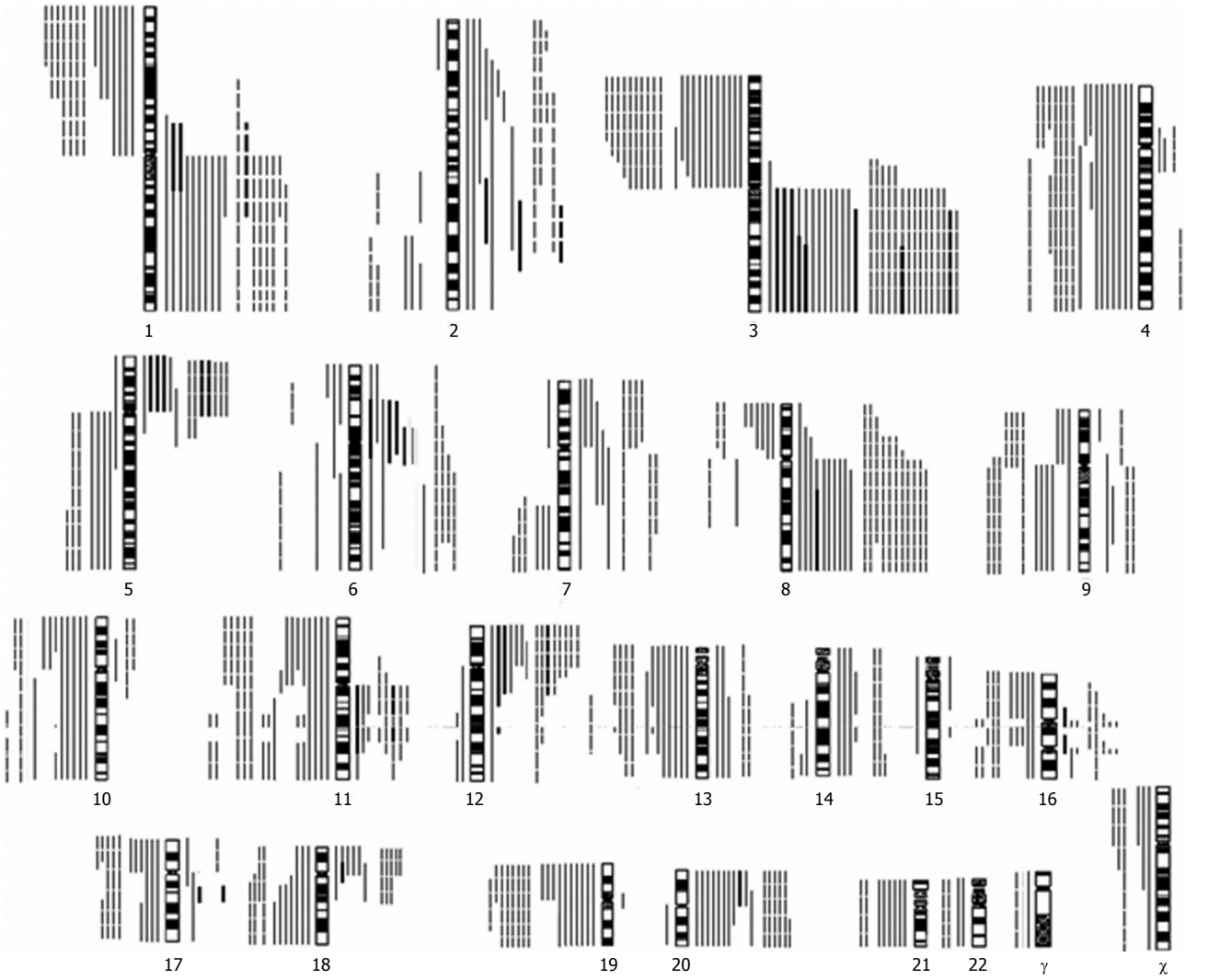

Figure 2 Copy number gains and losses in ESCC by CGH. Chromosomal localization of gains is on the right side of chromosome ideograms and that of losses are on the left side. Thick lines indicate amplified regions. Straight lines indicate lymph nodes metastasis. Conversely primary tumors are indicated 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 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.

Table 3 Chromosomal aberrations with primary ESCC and lymph node metastasis

Chromosomal changes	Primary tumor (%)	Lymph node metastasis %	*P* 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

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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S- Editor Zhu WL **L- Editor** Ma JY **E- Editor** Lu W

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World J Gastroenterol 2008 March 28; 14(12): 1836-1841
World Journal of Gastroenterology ISSN 1007-9327

VIRAL HEPATITIS

Effects of two novel nucleoside analogues on different hepatitis B virus promoters

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Received: August 6, 2007 Revised: December 19, 2007

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.

Key words: Hepatitis B virus; Nucleoside analogue; Hepatitis B virus promoter; Enhanced green fluorescent protein; Fluorescence activated cell sorter

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He XX, Lin JS, Chang Y, Zhang YH, Li Y, Wang XY, Xu D, Cheng XM. Effects of two novel nucleoside analogues on different hepatitis B virus promoters. *World J Gastroenterol* 2008; 14(12): 1836-1841 Available from: URL: http://www.wjgnet.com/1007-9327/14/1836.asp DOI: http://dx.doi.org/10.3748/wjg.14.1836

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 μmol/L, respectively) with little cytotoxicity (IC_{50} = 200 and 50 μmol/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 p3.6Ⅱ 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* (Dh5α) 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 p3.6Ⅱ, 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 lowercase 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 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℃ overnight. The ligated products were used to transform *E. coli* (Dh5α). 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.

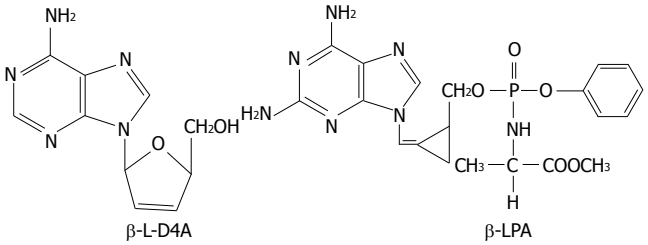

Figure 1 Structures of β-L-D4A and β-LPA.

Table 1 Primer sequences used for PCR

Target	Primer sequences	Product size (bp)
preSp	F5′-GACAAAggtaccAAACCATATTATCC-3′	229
	R5′-GAGGAccggtAACAGAAAGATTCGT-3′	
Sp	F5′-GATTGgtaccTCAACCCCAACAAG-3′	458
	R5′-GAAAAAccggtCCTGTAACACGAG-3′	
Cp	F5′-CCACCggtaccTGCCCAAGGTCTTA-3′	302
	R5′-TCCAAAccggtTATACGGGTCAATG-3′	
Xp	F5′-GTATggtaccGAATTGTGGGTCTTTTG-3′	445
	R5′-ACGTAAACAccggtCGTCCCGCGC-3′	

Expression assays

HepG2 cells were incubated in DMEM medium with 10% (vol/vol) FBS at 37℃ in a moist atmosphere containing 5% CO_2/95% 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 μg 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 μmol/L in 4 wells, 0.4 μmol/L in 4 wells, 2 μmol/L in 4 wells, 10 μmol/L in 4 wells). Four wells of cells were treated with lamivudine at 1 μmol/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 μL of PBS per well before being subjected to flow cytometry (Becton Dickinson) analysis. The same experiments were performed with β-LPA.

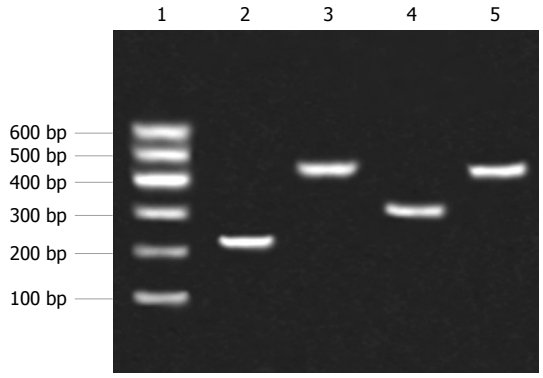

Figure 2 The HBV promoters obtained from PCR were separated by agarose gel electrophoresis. Lane 1: Marker; Lane 2: preSp; Lane 3: Sp; Lane 4: Cp; Lane 5: Xp.

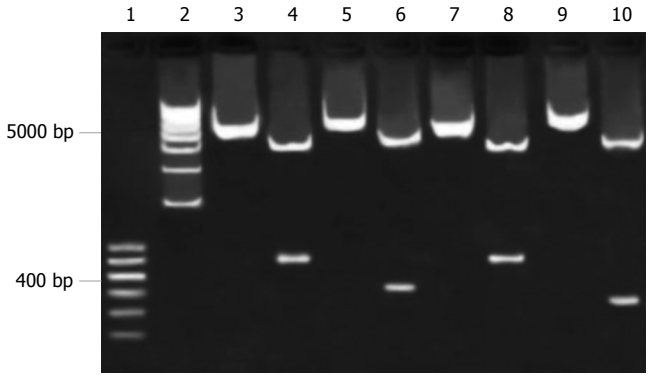

Figure 3 Restriction analysis of recombinant vectors. Lanes 1, 2: Marker; Lanes 3, 4: pEGFP-Sp before and after being digested by Acc65 I and Age I ; Lanes 5, 6: pEGFP-Cp before and after being digested by Acc65 I and Age I ; Lanes 7, 8: pEGFP-Xp before and after being digested by Acc65 I and Age I ; Lanes 9, 10: pEGFP-preSp before and after being digested by Acc65 I and Age I .

Statistical analysis

Data are expressed as mean ± 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 (p3.6Ⅱ). 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 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

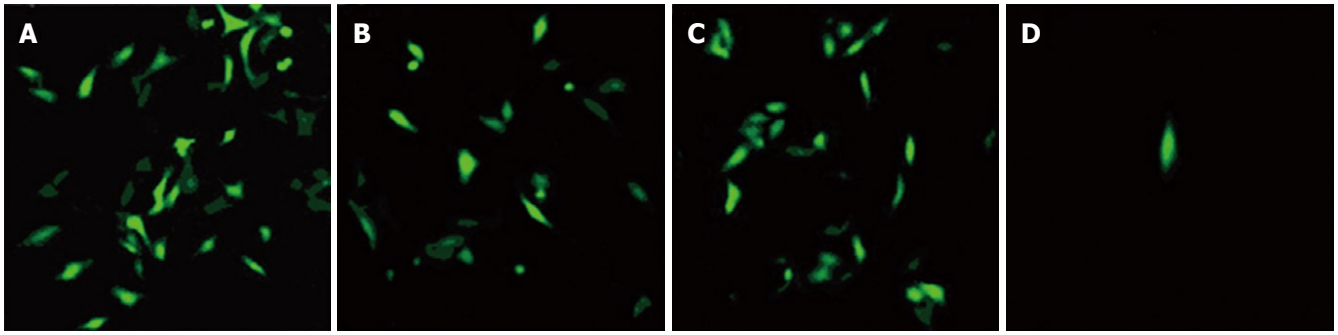

Figure 4 Forty-eight hours after transfection, EGFP positive cells were detected by fluorescence microscopy (× 100). **A**: HepG2 cells transfected with pEGFP-Sp; **B**: HepG2 cells transfected with pEGFP-Cp; **C**: HepG2 cells transfected with pEGFP-Xp; **D**: HepG2 cells transfected with pEGFP-preSp.

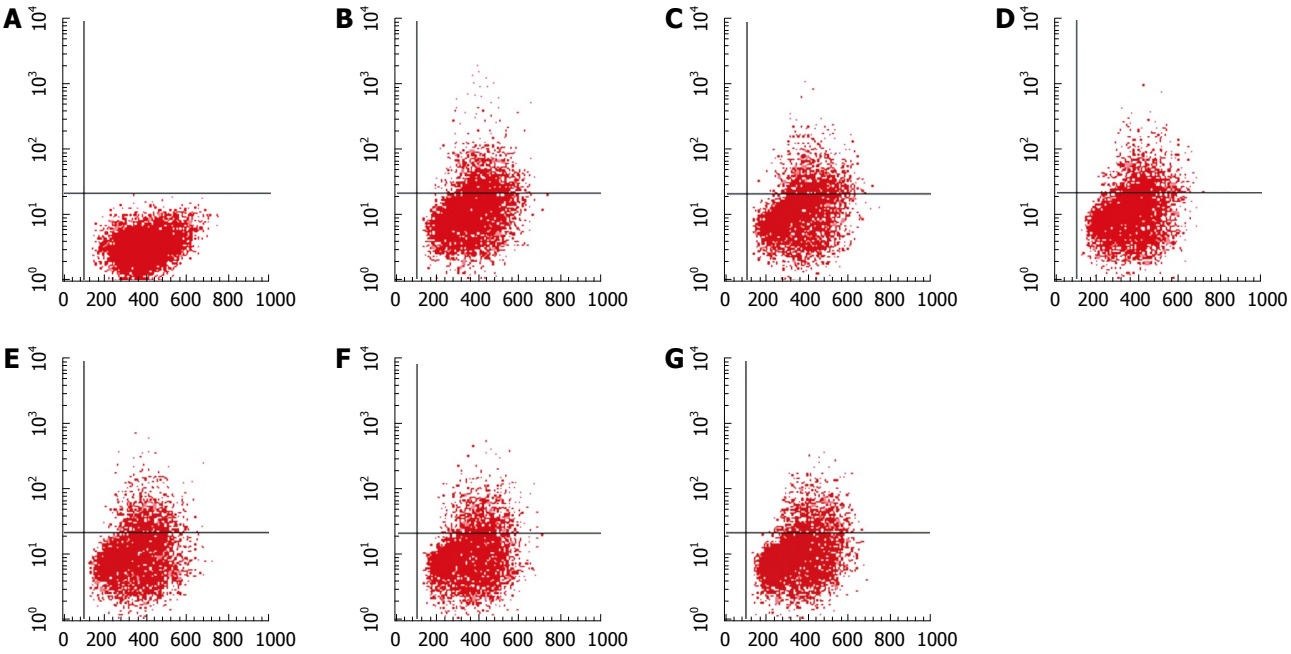

Figure 5 β-L-D4A inhibited the expression of EGFP in HepG2 cells transfected with pEGFP-Sp, as determined by FACS analysis. **A**: HepG2 cells not transfected with pEGFP-Sp, not treated with drug, the percentage of EGFP-positive cells was 0; **B**: HepG2 cells transfected with pEGFP-Sp, not treated with drug, the percentage was 21.42%; **C**: HepG2 cells transfected with pEGFP-Sp, treated with Lamivudine at 1 μmol/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 μmol/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 (μmol/L)	*n*	EGFP-positive cells (%) (mean ± SD)		Inhibition rate (%)	
		Sp	Xp	Sp	Xp
Control	3	21.26 ± 0.25	18.52 ± 1.25	0.0	0.0
(Lamivudine)	4	21.00 ± 0.43	18.37 ± 1.01	1.2	0.8
0.08	4	18.76 ± 0.41[b]	16.47 ± 0.49[b]	11.8	11.1
0.4	4	17.31 ± 0.41[b]	15.68 ± 0.36[b]	18.6	15.3
2	4	15.54 ± 0.48[b]	13.54 ± 0.59[b]	26.9	26.9
10	4	11.33 ± 0.32[b]	10.84 ± 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 (μmol/L)	*n*	EGFP-positive cells (%) (mean ± SD)		Inhibition rate (%)	
		Cp	Xp	Cp	Xp
Control	3	13.99 ± 0.29	18.58 ± 0.39	0.0	0.0
(Lamivudine)	4	13.80 ± 0.63	18.46 ± 0.49	1.3	0.6
0.002	4	12.98 ± 0.16[b]	17.54 ± 0.31[b]	7.2	5.6
0.01	4	11.96 ± 0.75[b]	16.18 ± 0.21[b]	14.5	12.9
0.05	4	10.88 ± 0.43[b]	14.63 ± 0.37[b]	22.2	21.3
1	4	8.79 ± 0.56[b]	11.94 ± 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 footprinting 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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S- Editor Zhu LH **L- Editor** Kerr C **E- Editor** Liu Y

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World J Gastroenterol 2008 March 28; 14(12): 1842-1850
World Journal of Gastroenterology ISSN 1007-9327

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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Supported by The Wilhelm Sander-Stiftung, No. 204.036.1
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Received: September 26, 2007 Revised: December 19, 2007

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 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.

Key words: Adenoviral vectors; Tumor vaccination; Fusogenic membrane protein; Colorectal cancer; Syngeneic tumor model

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Hoffmann D, Grunwald T, Bayer W, Wildner O. 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. *World J Gastroenterol* 2008; 14(12): 1842-1850 Available from: URL: http://www.wjgnet.com/1007-9327/14/1842.asp DOI: http://dx.doi.org/10.3748/wjg.14.1842

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 μg/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 (Δ524-574). 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 μL into the right hind flank and 1×10^4 cells in 100 μL 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 mm^3 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 μL 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 CO_2 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:

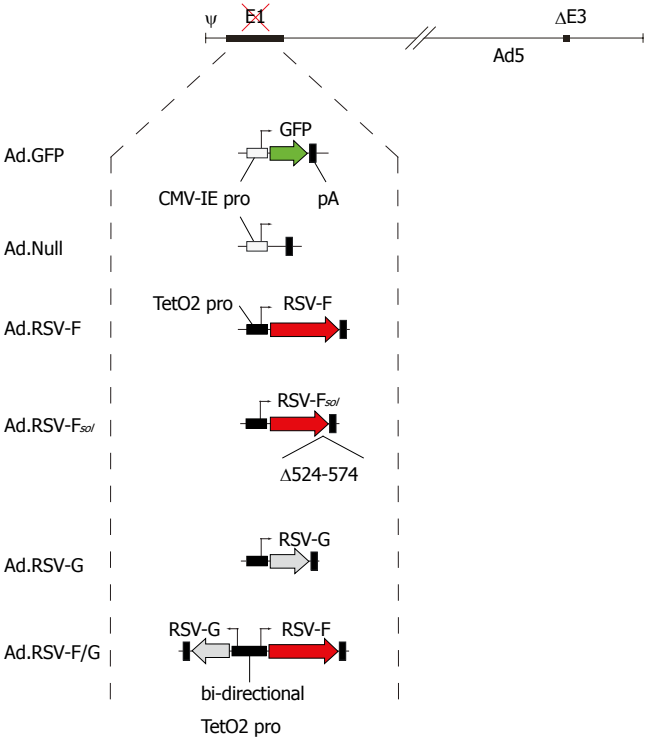

Figure 1 Adenovirus vector design. All adenovirus vectors used in this study are E1- and E3-deleted and Ad5-based. The vector Ad.GFP encodes green fluorescent protein and the vector Ad.Null served as an adenovirus vector control. The RSV glycoprotein encoding vectors carried the transgene under the transcriptional 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 (Δ524-574).

$$\text{Specific lysis (\%)} = \frac{\text{Experimental } A_{490} - \text{Effector spontaneous } A_{490} - \text{Target spontaneous } A_{490}}{\text{Target maximum } A_{490} - \text{Target spontaneous } A_{490}} \times 100$$

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

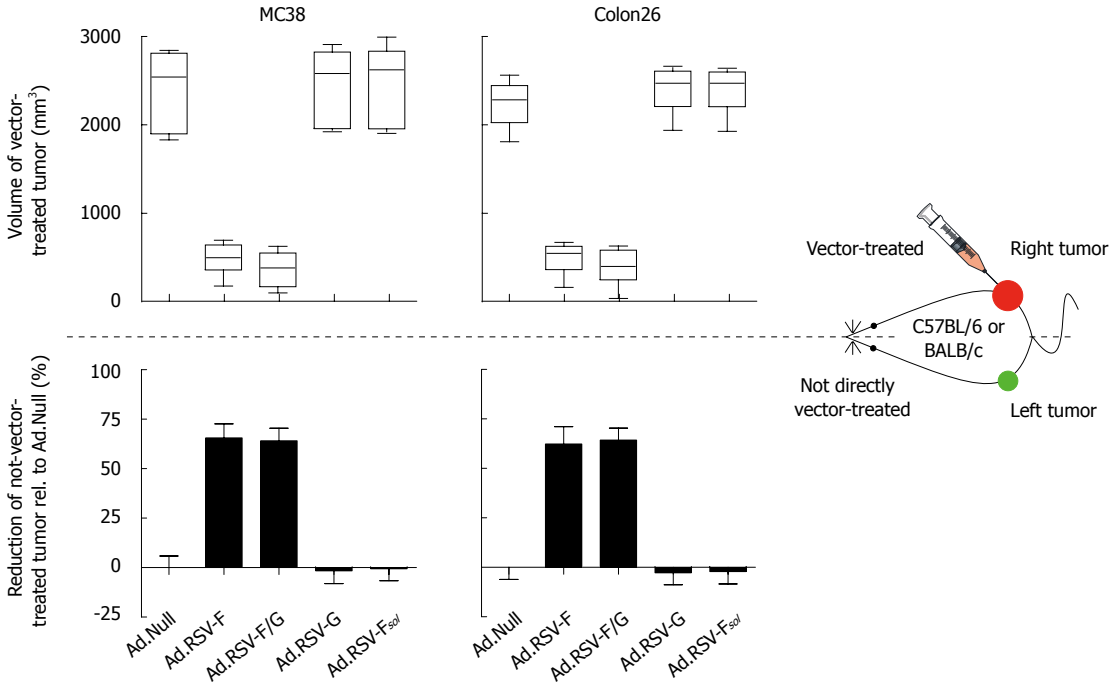

Figure 2 Intratumoral expression of RSV-F or RSV-F/G induces an anti-neoplastic effect. In a bilateral subcutaneous syngeneic MC38 or Colon26 colon cancer model, the indicated adenovirus vectors were inoculated on d 0 and d 2 into the tumor on the right flank. No viral vectors were inoculated into the 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 ± SD).

RSF-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 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 ($P < 0.001$).

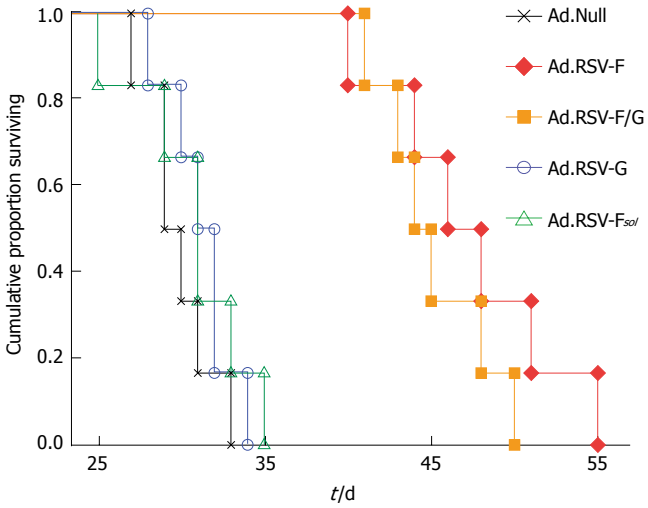

Figure 3 Kaplan-Meier survival analysis. In the bilateral subcutaneous syngeneic MC38 colon cancer model described in Figure 2, one tumor was treated with the indicated adenoviral vectors, and survival time was monitored up to d 55.

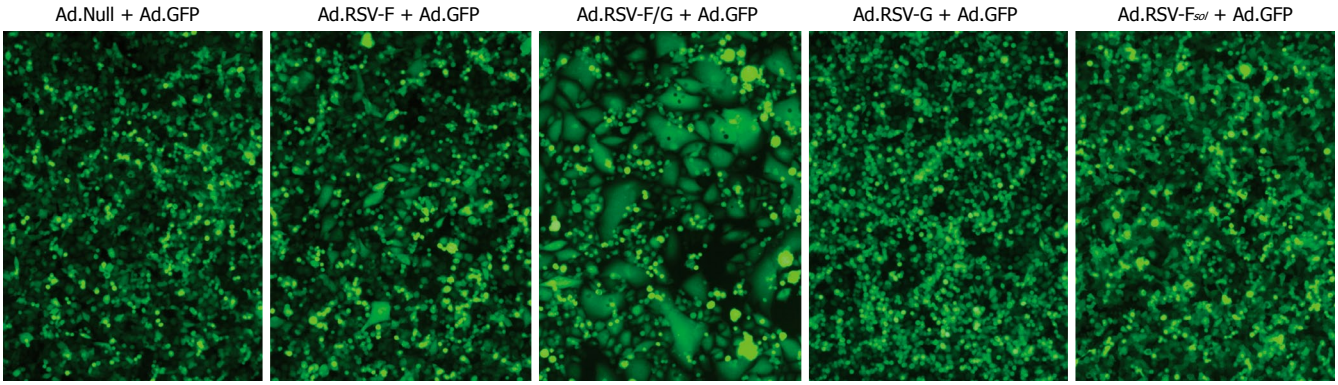

Figure 4 Fluorescence micrographs. MC38 cells were transduced *in vitro* with indicated RSV glycoprotein encoding adenoviruses and an adenovirus encoding GFP. Transduction with Ad.GFP enhanced the visibility of syncytia. Representative pictures 48 h after transduction are shown (× 200). Similar data were obtained with the Colon26 cells (data not shown).

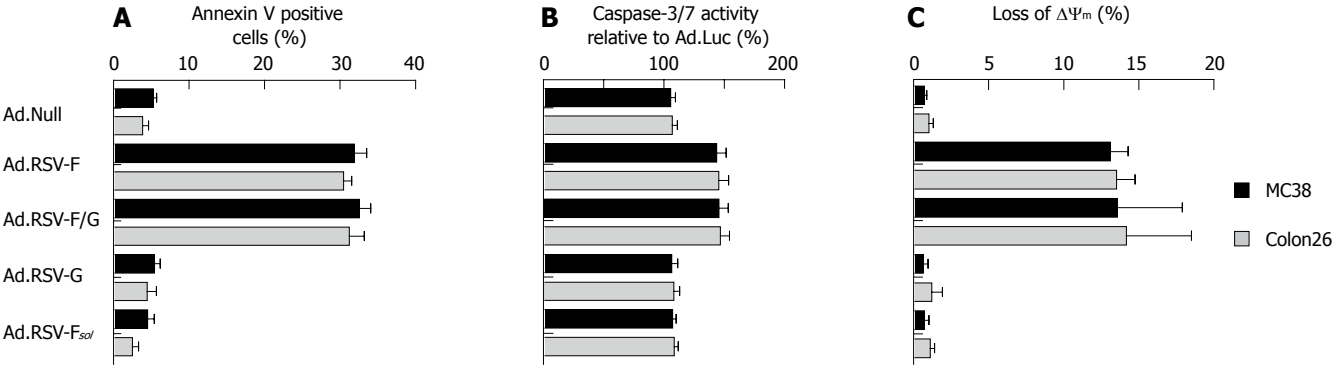

Figure 5 Analysis of apoptosis. **A**: MC38 or Colon26 cells were transduced *in vitro* with indicated adenovirus vectors and early apoptotic events were analyzed by flow cytometric measurements of phosphatidylserine translocation to the outer membrane by annexin V binding; **B**: Because apoptosis is essentially executed by proteases of the caspase family, we analyzed caspase-3/7 activity. Relative caspase activities are given as means ± 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

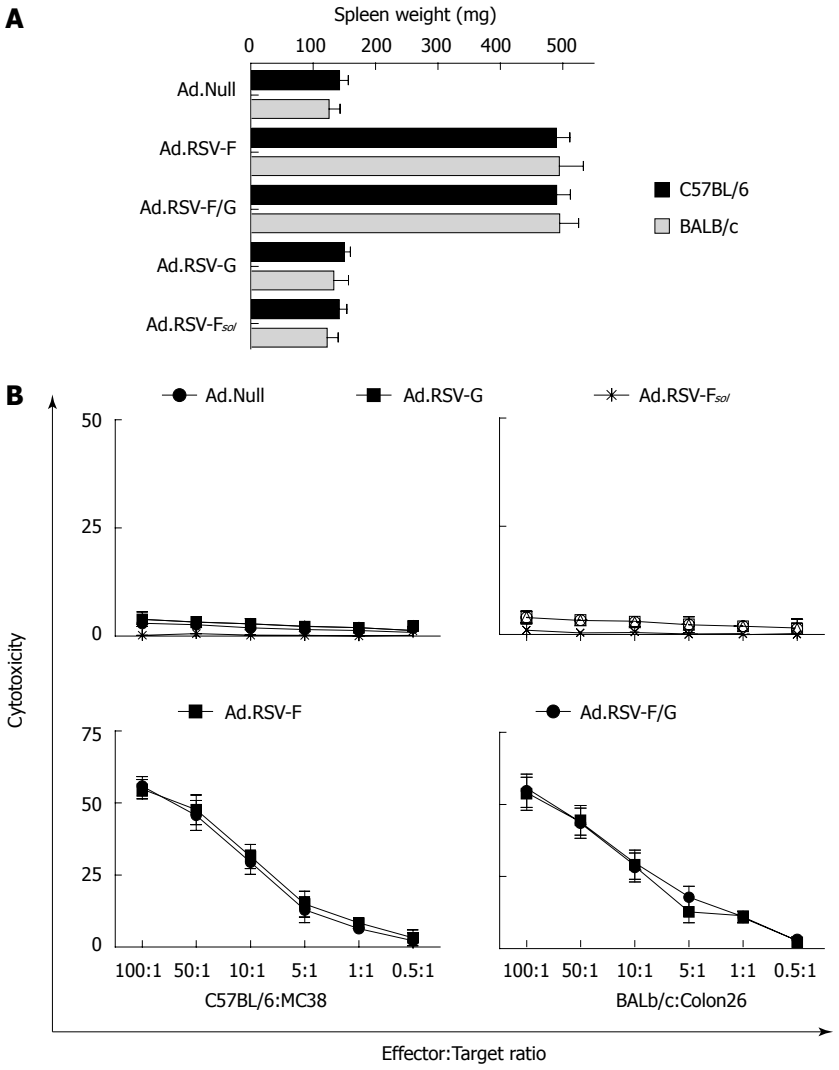

Figure 6 Effects of indicated treatments on the spleen weight and cytotoxic T cell induction. **A**: In the bilateral subcutaneous syngeneic MC38 and Colon26 tumor model described in Figure 2, animals were euthanized at day 28 and spleen weight was determined (mean ± SD); **B**: In addition, we determined 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 ± 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 90α[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 be 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.

ACKNOWLEDGMENTS

The authors are grateful to Steven A Rosenberg (Surgery Branch, NCI, National Institutes of Health, Bethesda, MD) for the MC38 cells. The authors would like to thank Klaus Überla for providing support and Cathrin Walter (West German Cancer Center University of Duisburg-Essen, Essen, Germany) for critical review of this manuscript.

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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World J Gastroenterol 2008 March 28; 14(12): 1851-1857
World Journal of Gastroenterology ISSN 1007-9327

BASIC RESEARCH

Effect of JIANPI HUOXUE decoction on inflammatory cytokine secretion pathway in rat liver with lipopolysaccharide challenge

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Supported by The National Natural Science Foundation of China, No.30371818; Shanghai Rising-Star Program, No. 07QA14052; Shanghai Leading Academic Discipline Project, Y0302 and Shanghai Educational Development Foundation, No. 2007CG56
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Received: October 6, 2007 Revised: January 15, 2008

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 μg/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-IκB) 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-IκB (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-IκB and endotoxin receptor TLR2.

Key words: JIANPI HUOXUE decoction; Lipopolysaccharide; Kupffer cell; Cytokine; Endotoxin receptor

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Peng JH, Hu YY, Cheng Y, Han C, Xu LL, Feng Q, Chen SD, Tao Q, Li HS, Li XM. Effect of JIANPI HUOXUE decoction on inflammatory cytokine secretion pathway in rat liver with lipopolysaccharide challenge. *World J Gastroenterol* 2008; 14(12): 1851-1857 Available from: URL: http://www.wjgnet.com/1007-9327/14/1851.asp DOI: http://dx.doi.org/10.3748/wjg.14.1851

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 macroce phala Koidz. Salvia miltiorrhiza Bge.*, *Citrus aurantium L.*, *Paeonia lzctiilora pall.*, *Pueraria lobata* (*willd.*) *Ohwi*, *Alisma orientalis* (*Sam.*) *Juzep*, *Schisandra chinensis* (*Turcz.*) *Baill.* and *Curuma longa L. Altractylodes macroce phala 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℃) 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 μg/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 μm 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-buffed saline (pH 7.4) and embedded in paraffin wax from which 4 μm 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℃ for 10 min. The specimens were treated with 0.6% hydrogen peroxide-methanol, following 30 min of endogenous peroxidase blockage at 37℃. 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℃ 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℃, diamino benzidine (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-κB (p-IκB) 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-IκB 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℃ 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 Shenggong Co.) used are described in Table 1.

Two-step PCR procedure was recommended as follows: pre-denaturation for 10 s at 95℃, 1 cycle; 95℃ for 5 s and 59℃ 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 ± 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'-TGACGAGGCCCAGAGCAAGA-3'(F)	331
	5'-ATGGGCACAGTGTGGGTGAC-3'(R)	
TNF-α	5'-GGCAGCCTTGTCCCTTGAAGAG-3'(F)	171
	5'-GTAGCCCACGTCGTAGCAAACC-3'(R)	
IL-6	5'-CCACTTCACAAGTCGGAGGCTTA-3'(F)	108
	5'-GTGCATCATCGCTGTTCATACAATC-3'(R)	
TLR4	5'-CTCACAACTTCAGTGGCTGGATTTA -3'(F)	178
	5'-TGTCTCCACAGCCACCAGATTC-3'(R)	
TLR2	5'-GGCCACAGGACTCAAGAGCA-3′(F)	102
	5'-AGAGGCCTATCACAGCCATCAAG-3'(R)	
CD14	5′- GAATCCCAGTCGGAGGCGTA-3′(F)	94
	5′-GGAGCAAAGCCAAAGTTCCTGA-3′(R)	

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 ± 9 007.03 *vs* 6031.70 ± 2296.56, $P < 0.05$) (Figure 2).

Effect of JHD on protein expression of CD68, p-IκB and TNF-α in liver tissues

After stimulation with LPS for 1.5 h, protein expression of CD68, p-IκB and TNF-α in liver tissues were increased significantly (CD68: 1.13 ± 0.49 *vs* 3.36 ± 1.69, $P < 0.05$; p-IκB: 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-IκB: 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, $P < 0.01$).

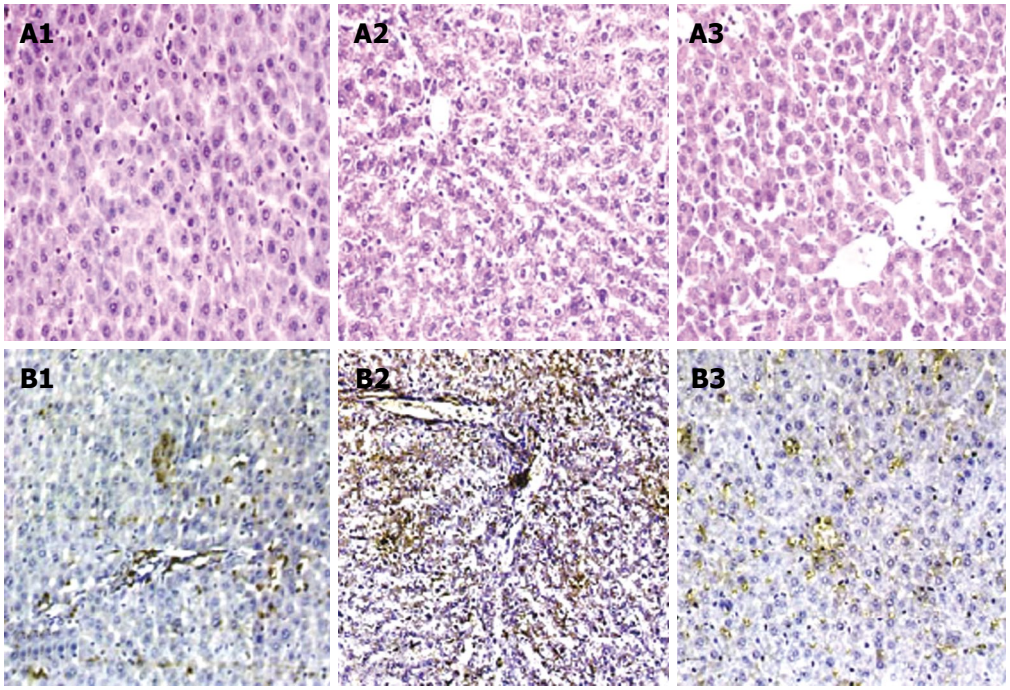

Figure 1 Effects of JHD on pathological changes and CD68 protein expression and distribution in liver tissue. **A**: HE stain (× 400); **A1**: Liver tissue in normal group; **A2**: Liver tissue in model group; **A3**: Liver tissue in JHD group; **B**: CD68 immunohistochemical stain (× 200); **B1**: Liver tissue in normal group; **B2**: Liver tissue in model group; **B3**: Liver tissue in JHD group.

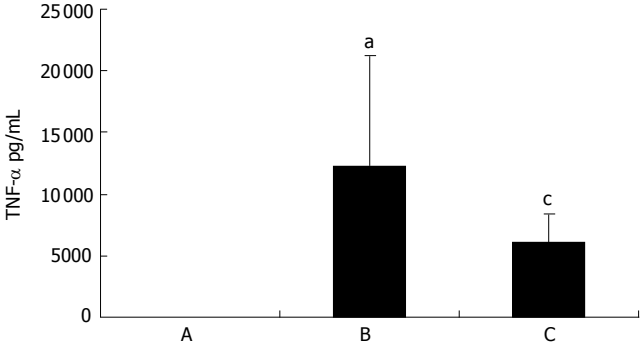

Figure 2 Effect of JHD on serum TNF-α level of rats. A: Normal group; B: Model group; C: JHD group. $^{a}P < 0.05$ *vs* A; $^{c}P < 0.05$ *vs* B.

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[26] and the gut-derived LPS consequently enter the circulation. Previous researches have confirmed that LPS promoted the phosphorylation of inhibit-κB (IκB), transferred the NF-κB 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-IκB 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 TLR-4[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 Fearns *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

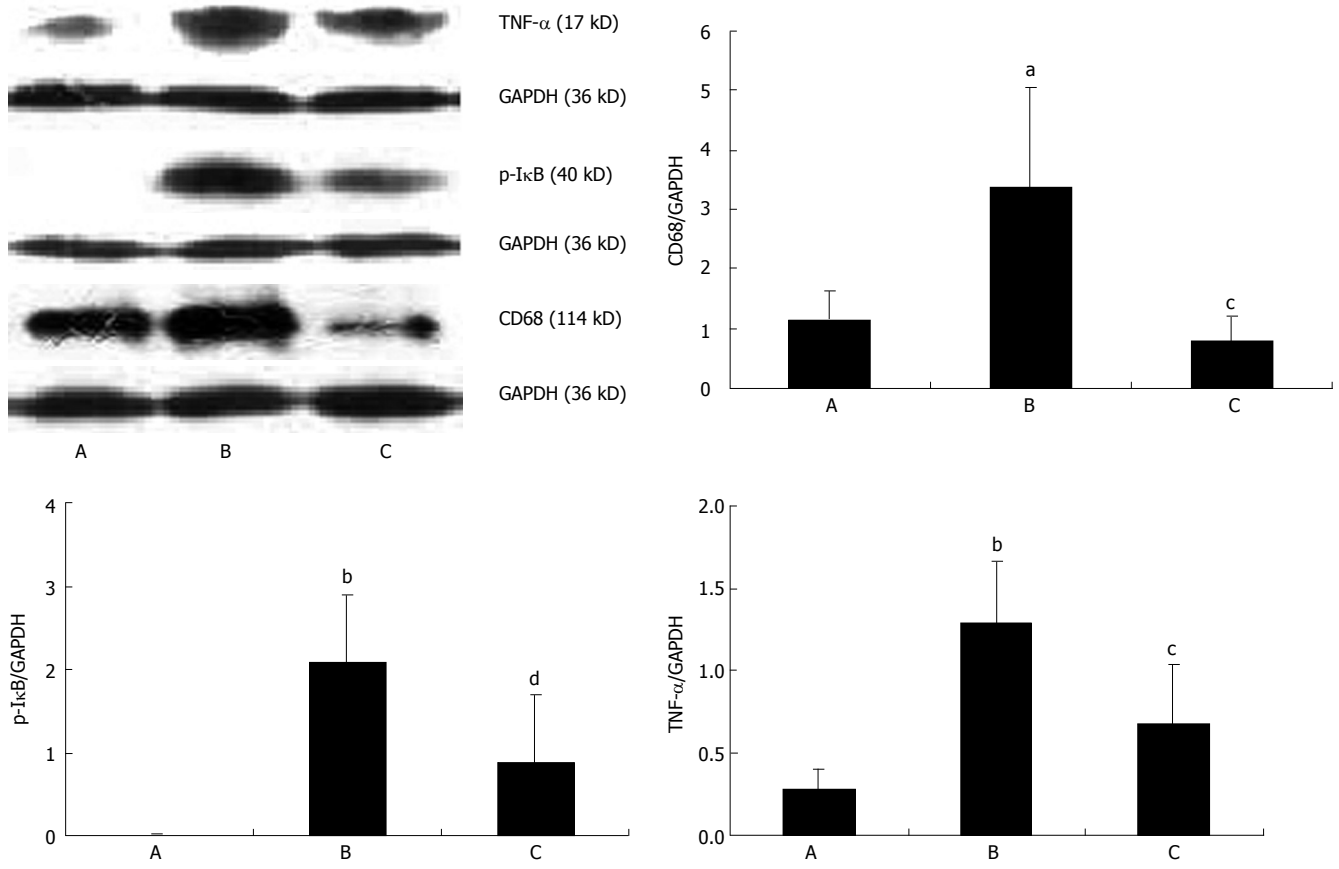

Figure 3 Effect of JHD on protein expression of CD68, p-IκB, TNF-α in liver tissue. A: Normal group; B: Model group; C: JHD group. [a]*P* < 0.05 *vs* A; [b]*P* < 0.01 *vs* A; [c]*P* < 0.05 *vs* B; [d]*P* < 0.01 *vs* B.

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 repurificated 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

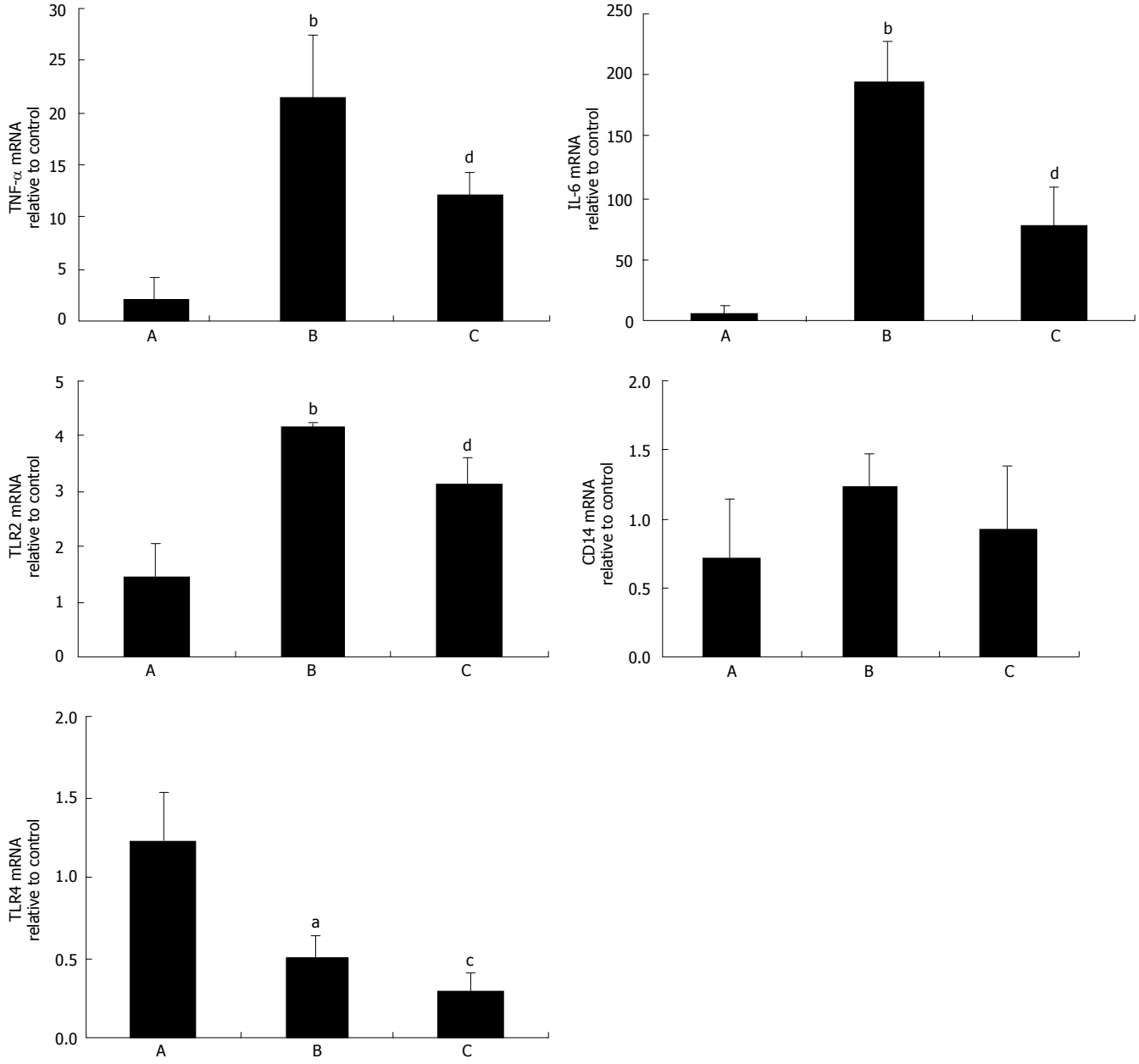

Figure 4 Effect of JHD on mRNA expression of TNF-α, IL-6, TLR2, TLR4, CD14 in liver tissue. A: Normal group; B: Model group; C: JHD group. [a]*P* < 0.05 *vs* A; [b]*P* < 0.01 *vs* A; [c]*P* < 0.05 *vs* B; [d]*P* < 0.01 *vs* B.

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-κB (p-IκB) 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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World J Gastroenterol 2008 March 28; 14(12): 1858-1865
World Journal of Gastroenterology ISSN 1007-9327

BASIC RESEARCH

Over-expressed and truncated midkines promote proliferation of BGC823 cells *in vitro* and tumor growth *in vivo*

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Supported by The Scientific Research Fund of Graduate School of Nanjing University, the Fund for Key Program of Ministry of Education, No. 02111 and the 985-Ⅱ Program of Nanjing University
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Received: November 7, 2007 Revised: December 10, 2007

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 turmorigenesis 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.

Key words: Midkine; Truncated midkine; Gastric cancer; BGC823; Tumorigenisis

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Wang QL, Wang H, Zhao SL, Huang YH, Hou YY. Over-expressed and truncated midkines promote proliferation of BGC823 cells *in vitro* and tumor growth *in vivo*. *World J Gastroenterol* 2008; 14(12): 1858-1865 Available from: URL: http://www.wjgnet.com/1007-9327/14/1858.asp DOI: http://dx.doi.org/10.3748/wjg.14.1858

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*.

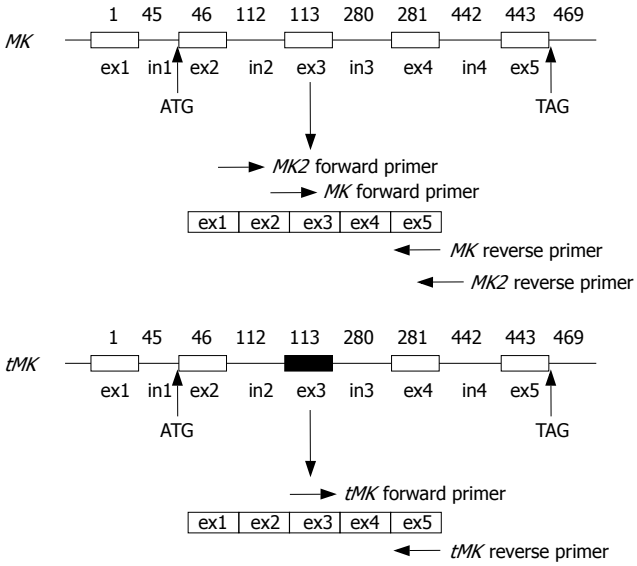

Figure 1 Illustration of *MK* and *tMK* gene DNA structures. Box: Exon (ex); Line: Intron (in); Shaded box; Truncated portion; ATG: Start site; TAG: Terminal site; Numeric figures: Nucleotide position of the mRNA transcript. Arrowheads indicate the sites of primer complemented with *MK* or *tMK* mRNA.

MATERIALS AND METHODS

Plasmids construction

Plasmids with *MK* and *tMK* eukaryotic expression were constructed[2,5,13] (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℃ under 5% humidified CO_2 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℃, 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 AidTM First Strand cDNA Synthesis Kit (Fermentas, USA). Subsequently, 2 μL 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℃), annealing (40 s at 55℃), and extension (30 s at 72℃). 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% NaN_3, 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	Excepted size (bp)	Cycles of PCR
MK-1 sense	AAAAAAGCTTATGAAAAAGAAAGATAAGGTGAAGAAG	Paul *et al*, 2001[13]	389	28
MK-1 antisense	AAAAGAATTCCTAGTCCTTTCCCTTCCCT			
tMK sense	AAAAAAGCTTATGAAAAAGAAAGCCGACTG		221	28
tMK antisense	AAAAGAATTCCTAGTCCTTTCCCTTCCCT			
MK-2 sense	ATGCAGCACCGAGGCTTCCT	Kaname *et al*, 1996[5]	447	30
MK-2 antisense	ATCCAGGCTTGGCGTCTAGT		279	
β-actin sense	CCACGAAACTACCTTCAACTC		270	28
β-actin antisense	TCATACTCCTGCTGCTTGCTGATCC			

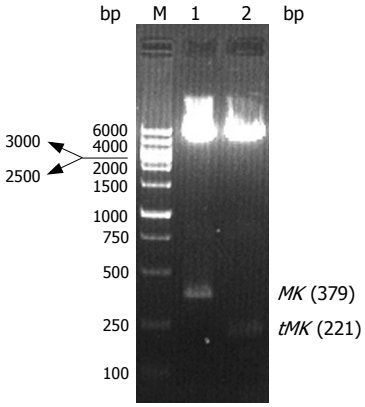

Figure 2 Restriction digestions of recombinant plasmids. M: Wide range DNA marker 100-6000 (TaKaRa); Lane 1: pcDNA3.1/*MK*; Lane 2: pcDNA3.1/*tMK*.

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 (Hynergy™ 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 2×10^3 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℃ in a humidified 5% CO_2. 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/*MK* 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 μL 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 mm^3 was calculated by the formula: Volume = $(\text{width})^2 \times \text{length}/2$. Tumor growth rates were calculated by the formula: TGR = $(V_{28th}-V_{21th})/7$ d. Data were presented as mean ± SE. Twenty-eight days after injection, nude mice were sacrificed, and the tumors were removed, photographed and weighed.

Immunohistochemistry

Immunostaining was performed on 6-μm 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% H_2O_2 in methanol for 30 min. Sections were exposed to rabbit polyclonal anti-*MK* antibody (1:250, Boster) overnight at 4℃. 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 ± 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

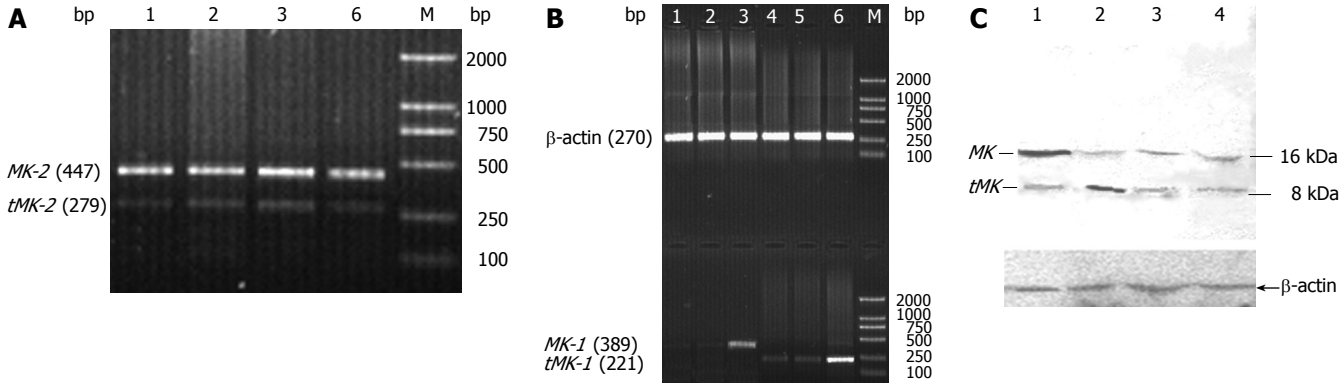

Figure 3 RT-PCR (**A**, **B**) and Western blotting (**C**) analysis of the expression of *MK* or *tMK* in BGC823 after transfection. **A** and **B**, M: DNA molecular weight standards, DL2000 (TaKaRa); Lane 1 and 4: BGC823; Lane 2 and 5: BGC823/vector; Lane 3: BGC823/*MK*; Lane 6: BGC823/*tMK*. **C**, 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 vivio*, respectively. To show that the upregulated *MK* and *tMK*

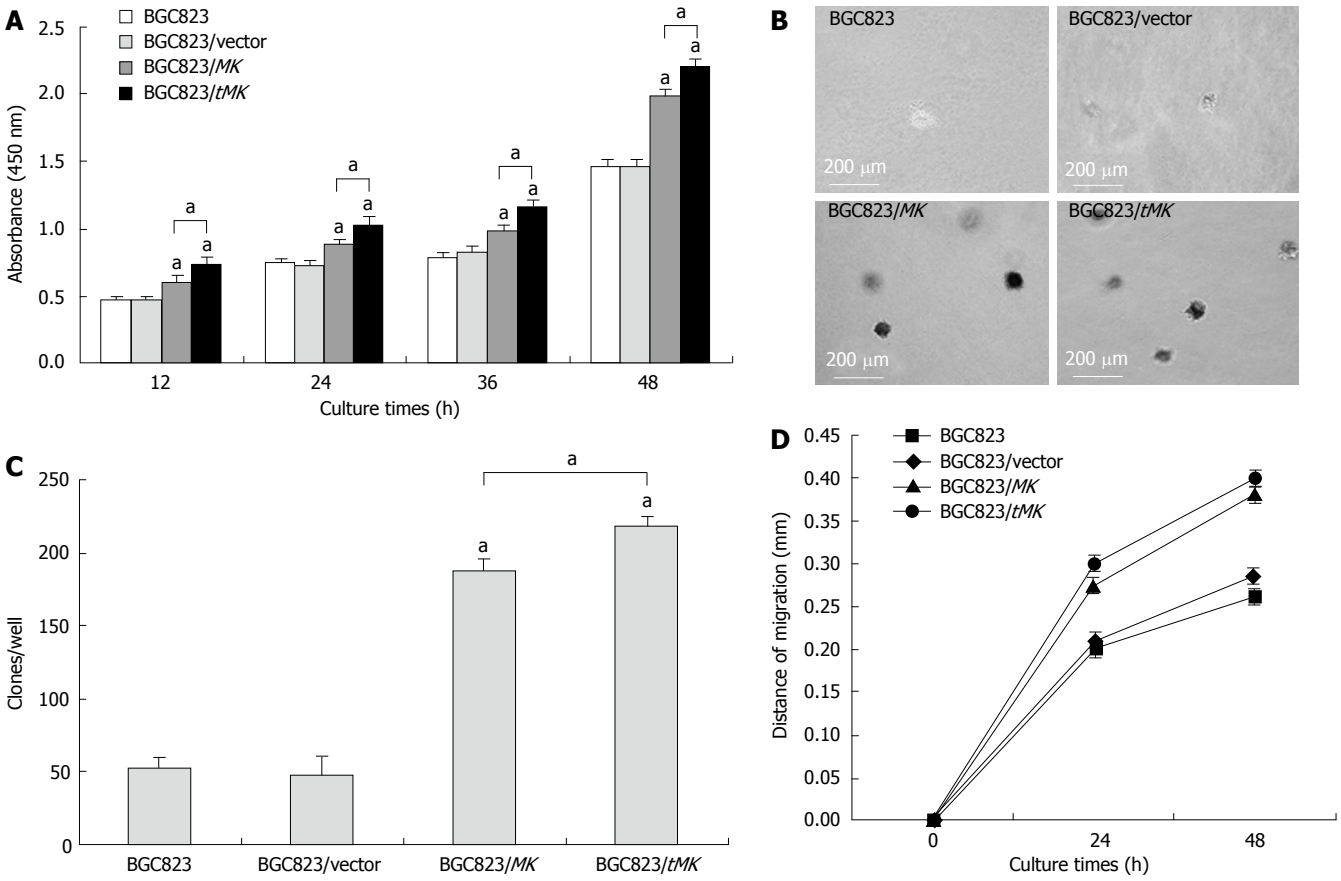

Figure 4 Effects of over-expressed *MK* or *tMK* on BGC823 cells *in vitro*. **A**: The cell proliferation determined by Cell Counting Kit ($P < 0.05$); **B**: Colony formation in soft agar observed under light microscope; **C**: Comparison of colony numbers. **D**: Analysis of cell migration.

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/*MK*	7	5 (71.43)[a]	6 (85.71)[a]	7 (100)	
BGC823/*tMK*	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

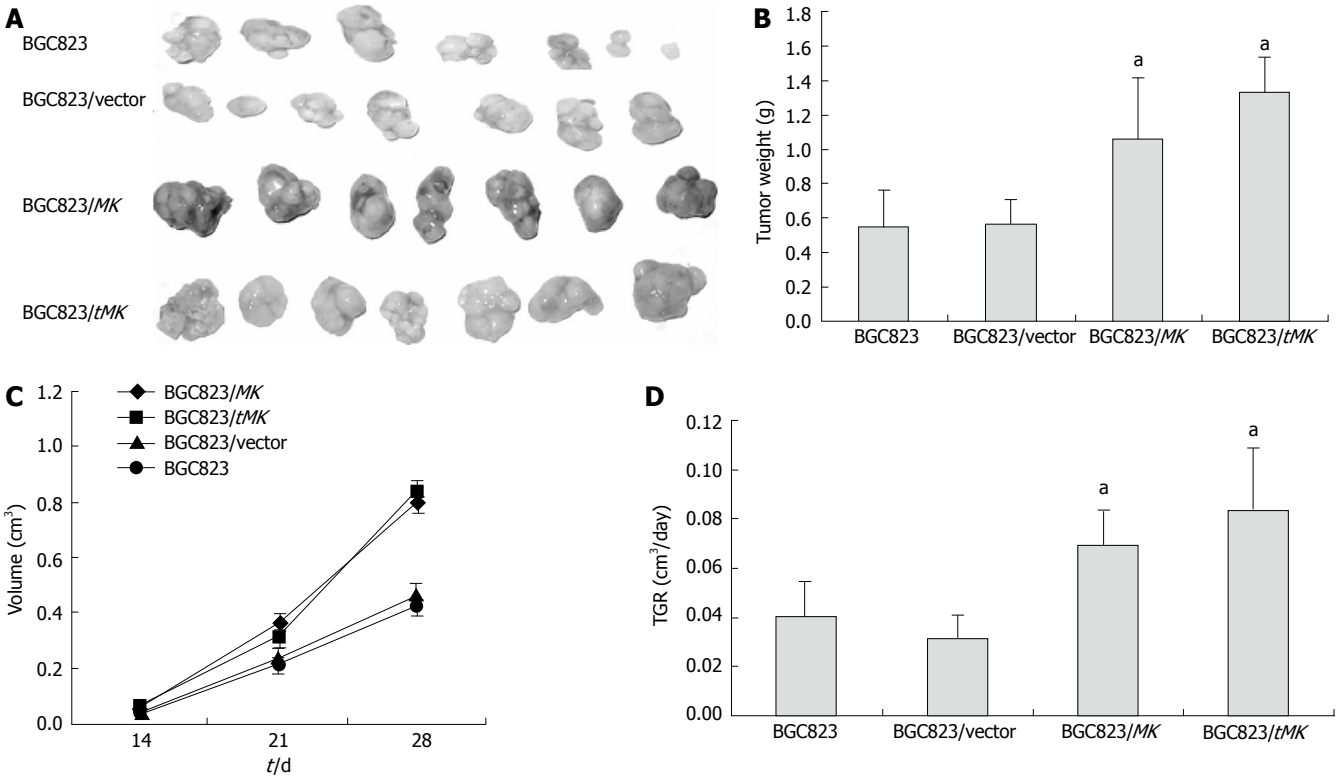

Figure 5 Promotion of tumorigensis of *MK*- or *tMK*- transfected cells *in vivo*. **A**: Photograph of tumor size; **B**: Comparison of tumor weight ($P < 0.05$); **C**: Measure of tumor volume ($^aP < 0.05$); **D**: Analysis of tumor growth rate ($^aP < 0.05$).

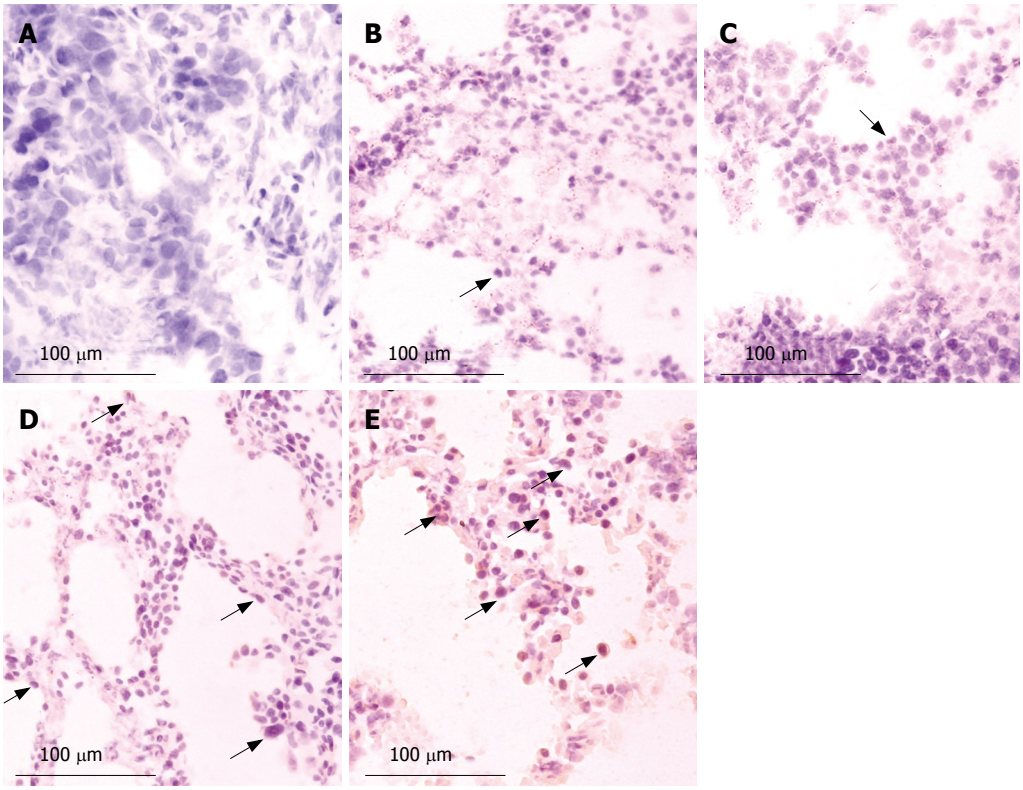

Figure 6 Immunohistochemical staining of tissues for *MK* and *tMK* with rabbit polyclonal anti-*MK* antibody. **A**: Negative control sections; **B**: Tumor tissue from BGC823 injected mice; **C**: Tumor tissue from BGC823/vector injected mice; **D**: Tumor tissue from BGC823/MK injected mice; **E**: Tumor tissue from BGC823/*tMK* injected mice (× 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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S- Editor Zhong XY **L- Editor** Ma JY **E- Editor** Lu W

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World J Gastroenterol 2008 March 28; 14(12): 1866-1871
World Journal of Gastroenterology ISSN 1007-9327

CLINICAL RESEARCH

Clinical and endoscopic features of Chinese reflux esophagitis patients

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Received: December 3, 2007 Revised: January 22, 2008

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.

Key words: Esophagitis; Los Angeles Classification; *Helicobacter pylori*; Hiatal hernia

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Li W, Zhang ST, Yu ZL. Clinical and endoscopic features of Chinese reflux esophagitis patients. *World J Gastroenterol* 2008; 14(12): 1866-1871 Available from: URL: http://www.wjgnet.com/1007-9327/14/1866.asp DOI: http://dx.doi.org/10.3748/wjg.14.1866

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

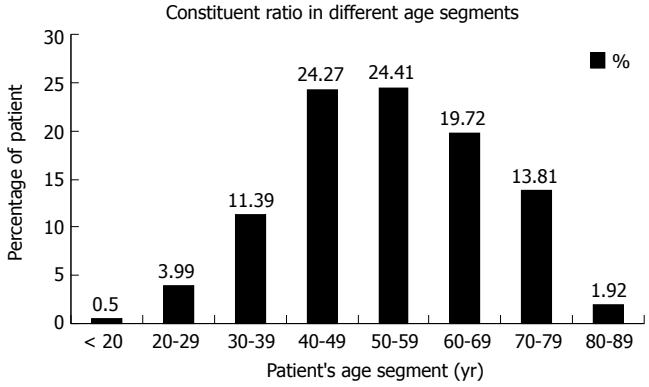

Figure 1 Percentage of patients at different age stages.

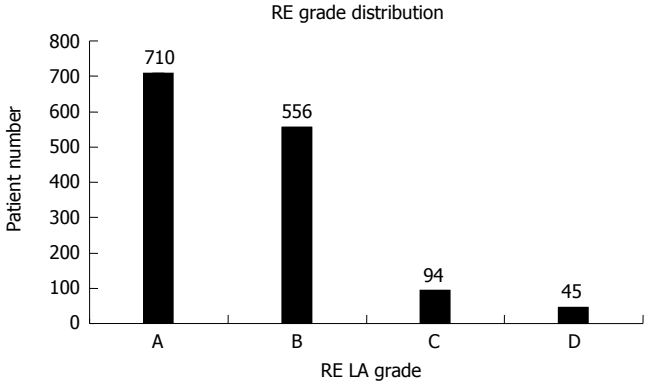

Figure 2 Distribution of disease grades in patients with reflux esophagitis.

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, C^{13} 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
H pylori+	7190	277	188	89	137	116	15	9
H pylori-	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

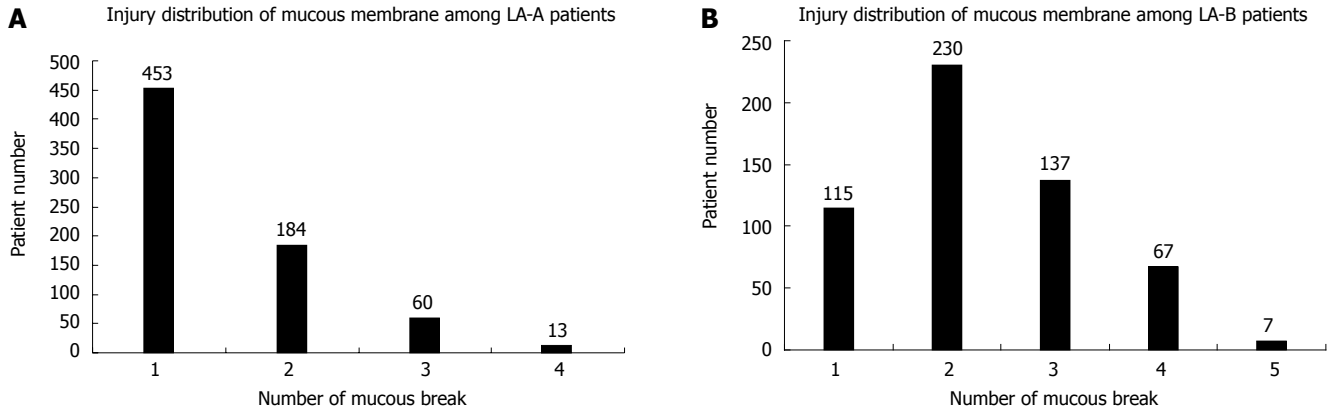

Figure 3 Distribution of mucosal damage in patients with Los Angeles Classification grade A (**A**) and grade B (**B**).

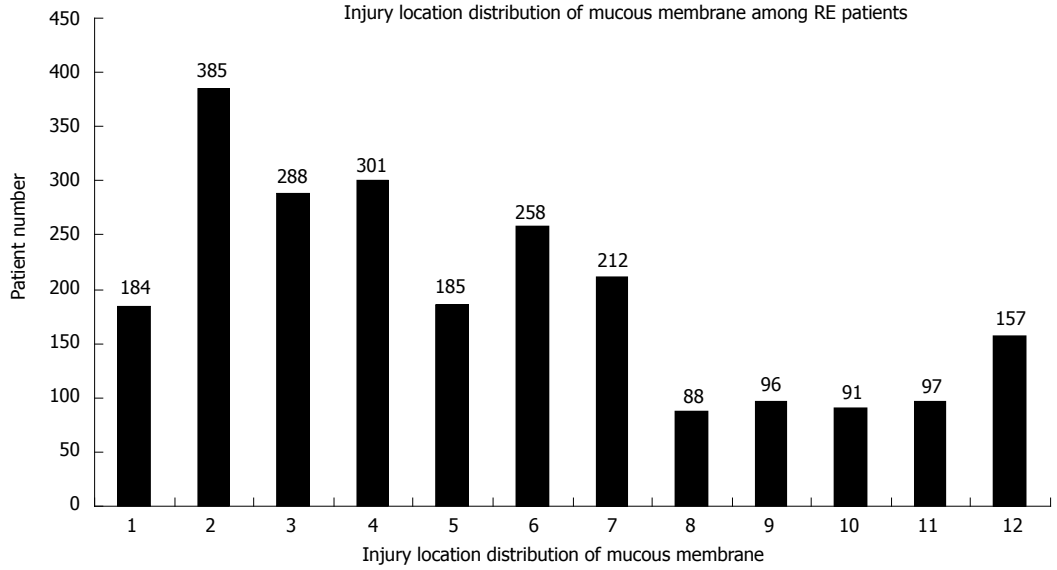

Figure 4 Distribution of location of mucosal damage in patients with reflux esophagitis.

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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S- Editor Zhong XY **L- Editor** Wang XL **E- Editor** Ma WH

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World J Gastroenterol 2008 March 28; 14(12): 1872-1877
World Journal of Gastroenterology ISSN 1007-9327

CLINICAL RESEARCH

Cost-effectiveness analysis of early veno-venous hemofiltration for severe acute pancreatitis in China

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Supported by The National Natural Science Foundation of China, No.30500684 and Clinical Research Foundation for Residency Granted by the Cooperative Project of West China Hospital and Daiichi Pharmaceutical Co. Ltd., the level I
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Received: August 19, 2007 Revised: December 14, 2007

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 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.

Key words: Veno-venous hemofiltration; Severe acute pancreatitis; Early management; Cost-effectiveness; Health economics

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Jiang K, Chen XZ, Xia Q, Tang WF, Wang L. Cost-effectiveness analysis of early veno-venous hemofiltration for severe acute pancreatitis in China. *World J Gastroenterol* 2008; 14(12): 1872-1877 Available from: URL: http://www.wjgnet.com/1007-9327/14/1872.asp DOI: http://dx.doi.org/10.3748/wjg.14.1872

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 ($\leqslant$ 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.

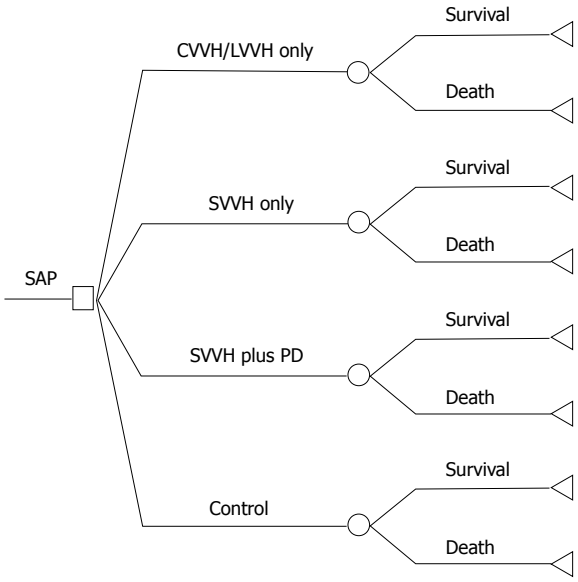

Figure 1 Decision tree of early hemofiltration for SAP.

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 ± 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	WMD
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)

[1]All the costs were converted to 2005 price before combination. [2]SVVH 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 (47 186 RMB), $7334 (56 122 RMB) and $5903 (45 172 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 $40 385 (30 8941 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.

Table 4 Results of cost-effectiveness analyses ($/%)

Subsets	CVVH/LVVH only	SVVH only	SVVH plus PD	Control
C/Survival	$22 122	$6167	$9224	$13 785
C/Complication prevention	$25 683	$7334	$9333	$19 247
C/Surgery preservation	$20 352	$5903	$8571	$16 030
ΔC/ΔSurvival	Dominated	-	Dominated	Dominated
ΔC/ΔComplication prévention	Dominated	-	$40 375	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.

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 (17 286 RMB) - $10 399 (79 570 RMB) and $5114 (39 130 RMB) - $12453 (95 286 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 $10 399 (79 570 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 imbalance between pro- and anti-inflammatory cytokines, resulting in the development of SIRS. Some mediators, such as TNF-alpha, 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-beta, 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

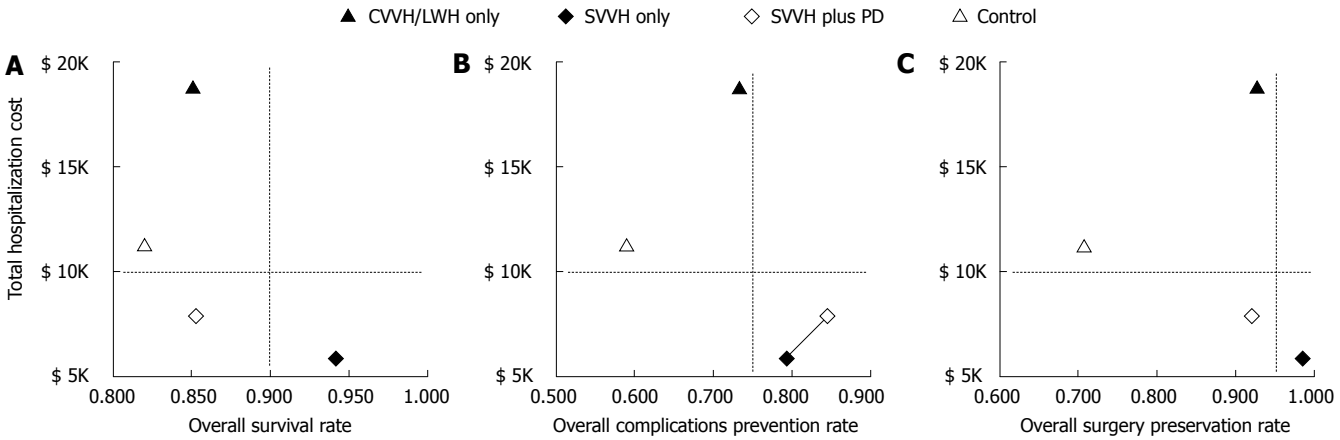

Figure 2 Cost-effectiveness analysis plots. **A**: Cost-survival ratio; **B**: Cost-complications prevention ratio; **C**: Cost-surgery preservation ratio.

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/LVVH only, SVVH plus PD and non-hemofiltration modalities. The CVVH/LVVH 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 ($\geqslant$ 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.

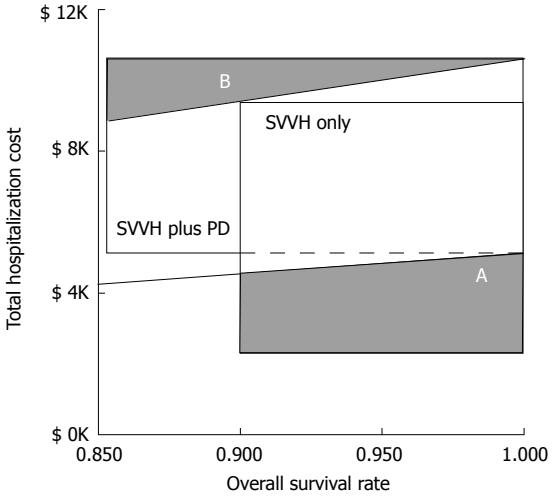

Figure 3 Two way sensitivity analysis of C/E ratio between SVVH only and SVVH plus PD modalities.

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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S- Editor Ma L **L- Editor** Anand BS **E- Editor** Lu W

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World J Gastroenterol 2008 March 28; 14(12): 1878-1884
World Journal of Gastroenterology ISSN 1007-9327

RAPID COMMUNICATION

Factors influencing a low rate of hepatitis C viral RNA clearance in heroin users from Southern China

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Received: January 11, 2007 Revised: February 5, 2008

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.

Key words: Hepatitis C virus; Clearance; Heroin users

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Garten RJ, Lai SH, Zhang JB, Liu W, Chen J, Yu XF. Factors influencing a low rate of hepatitis C viral RNA clearance in heroin users from Southern China. *World J Gastroenterol* 2008; 14(12): 1878-1884 Available from: URL: http://www.wjgnet.com/1007-9327/14/1878.asp DOI: http://dx.doi.org/10.3748/wjg.14.1878

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℃. 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
	317 (91.35)	30 (8.65)	347
Factor			
Location			
Binyang	200	20	220
Pingxiang	117	10	127
Mean age (yr)			
Mean ± SD	27.5 ± 5.7	25.4 ± 4.52	27.4 ± 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 ± SD	63.7 ± 26.4	54.72 ± 28.2	62.9 ± 26.6
Range	(9.0-165.2)	(13.1-124.2)	(9.0-165.2)
Mean frequency of drug use			
Per Month ± SD	73.9 ± 44.7	79.6 ± 34.6	74.4 ± 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 immuno-compromised (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 Adjusted OR (95% CI)	Final multivariate model Adjusted OR (95% CI)	*P*
Age (yr)				
⩽ 25	125 (13.6)	1.0	1.0	
> 25	222 (5.9)	0.472 (0.221-1.006)	0.400 (0.176-0.909)	0.029
HIV Ab status				
Negative	257 (10.5)	1.0	1.0	
Positive	90 (3.3)	0.294 (0.087-0.993)	0.256 (0.071-0.924)	0.038
HBsAg status				
Negative	301 (5.3)	1.0	1.0	
Positive	46 (30.4)	7.793 (3.484-17.430)	8.436 (3.646-19.520)	< 0.0001
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[3]. 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	Number tested	HCV RNA clearance rate (%)	Percent HIV Ab+	Percent HBsAg+
This study	Chinese IDU	347	8.7[1]	25.9%	13.3%
Thomas[3]	African American IDU	729	9.3		
	Non-African American IDU	44	36.0		
	Overall	773	10.9[1]	45.7%	3.5%
Alric[5]	Caucasian French	123	25[1]	ND	ND
Minton[6]	Caucasian English	172	20.3[1]	ND	ND
Yee[7]	English Hemophiliacs	200	14[1]	40.3%	ND
Alter[8]	American blood Donors (42% IDU)	248	14[2]	ND	ND
Kenny-Walsh[9]	Irish women	704	44.6[2]	ND	ND
Alter[10]	Non-Hispanic African American	196	14.0		
	Non-Hispanic Caucasian	119	32.0		
	Hispanic Americans NHANES	132	26.0		
	Overall	447	26.1[2]	ND	ND
Sagnelli[11]	Italian liver Patients	336	21.7[3]	ND	28%

[1]HCV RNA by Roche AMPLICOR HCV Qualitative Assay-Sensitivity > 50 IU/mL; [2]HCV RNA by In-house RT-PCR- Sensitivity Unknown; [3]HCV 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.

ACKNOWLEDGMENTS

We would like to thank Lara Sanders at Abbott Diagnostics for her assistance in performing the HCV serotyping.

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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S- Editor Zhu LH **L- Editor** Alpini GD **E- Editor** Ma WH

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World J Gastroenterol 2008 March 28; 14(12): 1885-1890
World Journal of Gastroenterology ISSN 1007-9327

RAPID COMMUNICATION

Effect of infliximab on small bowel stenoses in patients with Crohn's disease

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Received: September 20, 2007 Revised: January 19, 2008

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 during maintenance therapy.

Key words: Crohn's disease; Infliximab; Intestinal stenosis

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Pallotta N, Barberani F, Hassan NA, Guagnozzi D, Vincoli G, Corazziari E. Effect of infliximab on small bowel stenoses in patients with Crohn's disease. *World J Gastroenterol* 2008; 14(12): 1885-1890 Available from: URL: http://www.wjgnet.com/1007-9327/14/1885.asp DOI: http://dx.doi.org/10.3748/wjg.14.1885

INTRODUCTION

The effect of infliximab on symptomatic Crohn's intestinal stenosis is controversial and, even though there is no direct evidence indicating anti-TNFα 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 Inclusion	CDAI Last	FU months	Infusions[2] number	Stenosis 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; [1]Age at diagnosis; [2]Some infusions were delayed for intercurrent events (e.g. pregnancy, infections *etc*); [3]Stricturoplasty after 6 infusions of infliximab, in maintenance therapy; [4]Patients submitted to surgery during infliximab therapy; [5]Number 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

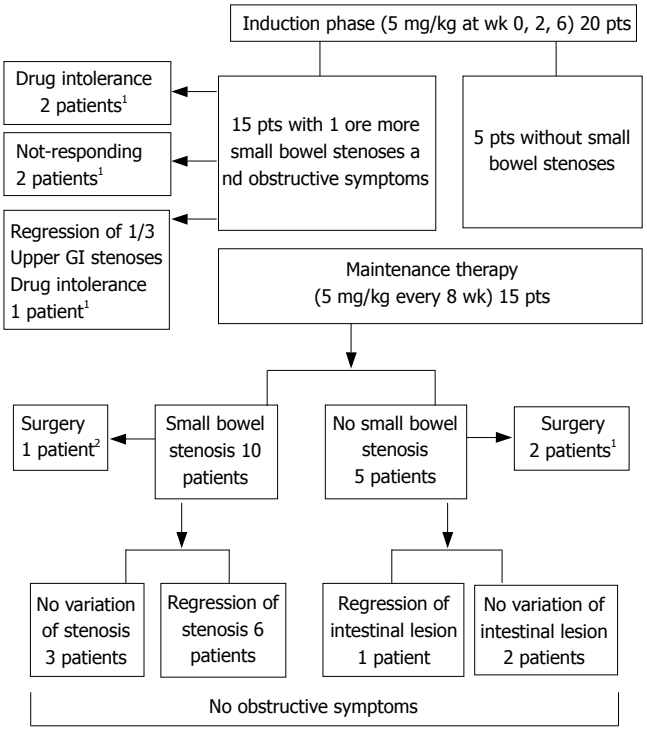

Figure 1 Table showing follow-up of patients on therapy with infliximab. [1]Stopped treatment; [2]In maintenance treatment after surgery.

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 temporaneous 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 mo 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

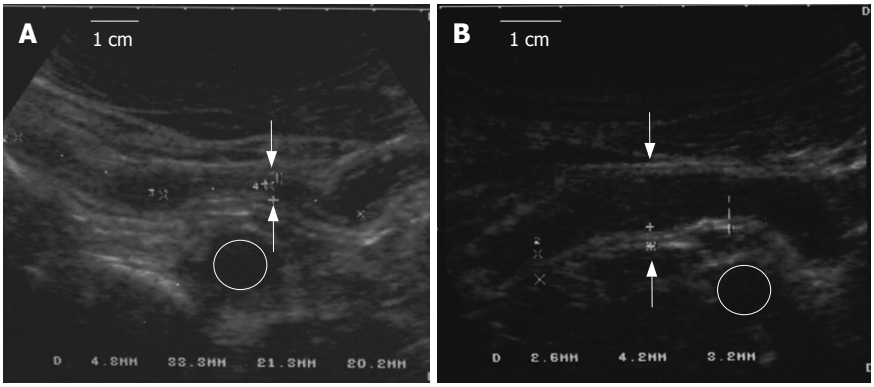

Figure 2 **A**: Before infliximab therapy: A stenotic lesion of the terminal ileum, with narrow lumen (white arrows) and increased thickness of the intestinal wall. The markers assess the length. The iliac vessel is indicated (circle); **B**: After infliximab therapy (22 cycles): The same ileal segment 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-κB[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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World J Gastroenterol 2008 March 28; 14(12): 1891-1897
World Journal of Gastroenterology ISSN 1007-9327

RAPID COMMUNICATION

KIT exon 11 codon 557/558 deletion/insertion mutations define a subset of gastrointestinal stromal tumors with malignant potential

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Supported by The Hellenic State Scholarship Foundation, Dept of Science Promotion, 2005 Grant for Scientific Research. No. 19366/2005
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Received: September 10, 2007 Revised: January 10, 2008

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.

Key words: Gastrointestinal stromal tumors; KIT gene; Platelet derived growth factor receptor alpha; Mutations; Malignant

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Kontogianni-Katsarou K, Dimitriadis E, Lariou C, Kairi-Vassilatou E, Pandis N, Kondi-Paphiti A. KIT exon 11 codon 557/558 deletion/insertion mutations define a subset of gastrointestinal stromal tumors with malignant potential. *World J Gastroenterol* 2008; 14(12): 1891-1897 Available from: URL: http://www.wjgnet.com/1007-9327/14/1891.asp DOI: http://dx.doi.org/10.3748/wjg.14.1891

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 mm^2, 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 assigned to risk assessment categories based on size, mitotic index, and location according to published criteria[17].

Table 1 Primer sequences used for KIT and PDGFRA PCR

Kit exon 9F tttggaaagctagtggttca	Kit exon 9R atggtagacagagcctaaac
Kit exon11F ctatttttccctttctcccc	Kit exon11R tacccaaaaaggtgacatgg
Kit exon 13F cttgacatcagtttgccag	Kit exon 13R aaaggcagcttggacacggcttta
Kit exon 17F tttctcctccaacctaatag	Kit exon 17R cctttgcaggactgtcaagc
PDGFRA exon 12aF	PDGFRA exon 12aR
ccagttacctgtcctggtcat	tggaaactcccatcttgagtc
PDGFRA exon 12bF	PDGFRA exon 12bR
aaattcgctggagggtcatt	ggaggttaccccatggaagt
PDGFR exon 18F	PDGFRA exon 18R
agtgtgtccaccgtgatctg	gtgtgggaagtgtggaggta

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, Glostroup, Danemark). 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℃ and for the KIT exon 13 and 17 primer sets 56℃, 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 PDFGRA 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 (× 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.

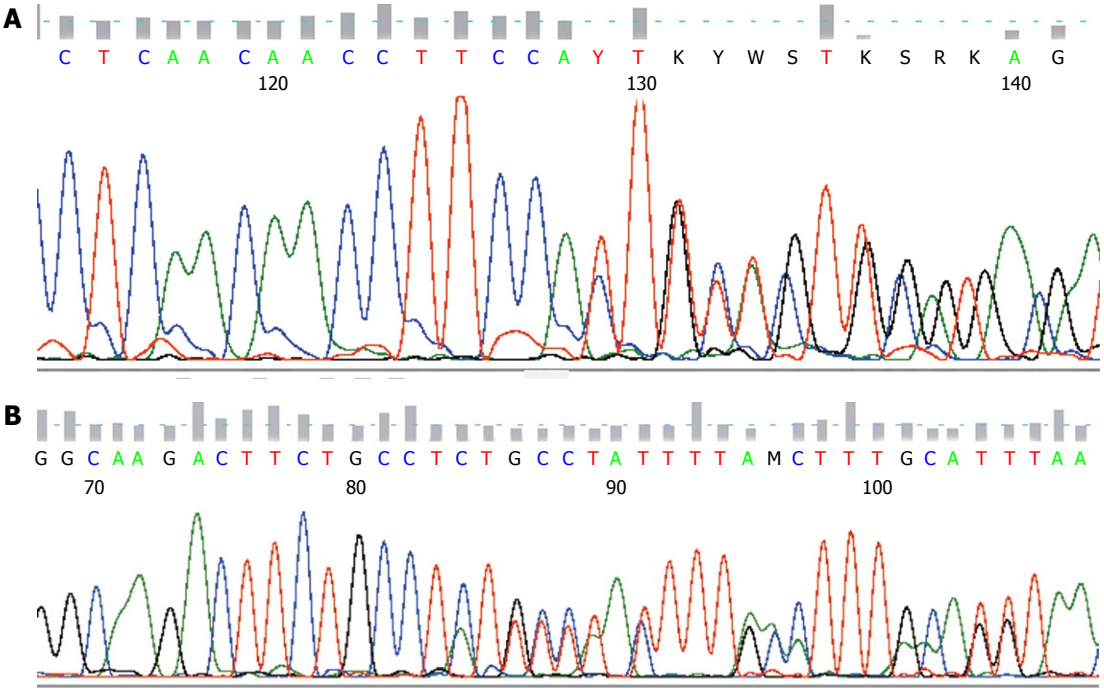

Figure 2 The sequencing data from a GIST. **A**: Showing KIT exon 11 deletion (pP551-Q556del); **B**: Showing KIT exon 9 insertion (p.A502-Y503insAS).

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	pV560D	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	pV560D	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 (≤ 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				
	Epithelioid (%)	Spindle cells (%)	Mixed type 1 (%)	Mixed type 2 (%)	*P*
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					
	Benign (%)	Very low malignant potential (%)	Low malignant potential (%)	Low moderate malignant potential (%)	Malignant (%)	*P*
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.

ACKNOWLEDGMENTS

We are grateful to Eugenia Tsompanaki, MSs, Department of Statistics, Athens University, School of Economics and Business, for her invaluable help in data management.

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.

Reasearch 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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World J Gastroenterol 2008 March 28; 14(12): 1898-1902
World Journal of Gastroenterology ISSN 1007-9327

RAPID COMMUNICATION

Portal hemodynamics as predictors of high risk esophageal varices in cirrhotic patients

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Received: October 6, 2007 Revised: December 4, 2007

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 (± SD) was 47.5 (± 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 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.

Key words: Liver cirrhosis; Doppler ultrasound; Portal hemodynamics; Esophageal varices; Prediction

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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 = (systolic velocity - end diastolic velocity)/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 (hepatic artery RI*0.69)*(splenic artery R*0.87)/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 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.

Table 1 Baseline characteristics of 85 cirrhotic patients [as *n* (%) or mean ± 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 (± 3.4)
Gastric varices	11 (12.9)
Portal hypertrophic gastropathy	75 (88.2)
Portal vein diameter (mm)	13.5 (± 2.4)
Splenic axis (cm)	15.7 (± 3.1)
Portal vein flow (cm/s)	14.6 (± 4.8)
Splenic artery resistance	0.7 (± 0.1)
Hepatic artery resistance	0.7 (± 0.1)

RESULTS

Eighty five consecutive cirrhotic patients (43 men, 42 women) were enrolled in the study. Mean age (± SD) of the study population was 47.5 (± 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 ($P \leqslant 0.0005$) 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 ± 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 ± 3.66	20.25 ± 5.05	< 0.0005	0.113
Portal vein diameter (mm)	13.88 ± 2.42	12.00 ± 1.69	0.004	0.242
Hepatic artery resistance index	0.73 ± 0.07	0.66 ± 0.07	0.001	0.210
Splenic artery resistance index	0.73 ± 0.06	0.62 ± 0.08	< 0.0005	0.168
Spleen size (cm)	15.98 ± 3.01	14.76 ± 3.66	0.166	0.431
Liver vascular index	8.31 ± 2.72	17.08 ± 6.28	< 0.0005	0.114
Congestion index	0.11 ± 0.03	0.06 ± 0.03	< 0.0005	0.128
Portal hypertensive index	2.62 ± 0.79	1.33 ± 0.53	< 0.0005	0.072

EV: Esophageal varices; AUC: Area under the curve.

Table 3 mean ± 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 ± 2.59	15.26 ± 5.06	0.001	0.715
Portal vein diameter (mm)	14.54 ± 1.48	13.24 ± 2.55	0.037	0.748
Hepatic artery resistance index	0.80 ± 0.06	0.70 ± 0.06	0.003	0.820
Splenic artery resistance index	0.76 ± 0.11	0.69 ± 0.06	< 0.0005	0.656
Spleen size (cm)	17.62 ± 3.05	15.21 ± 2.99	0.003	0.724
Liver vascular index	6.48 ± 2.78	10.96 ± 5.05	< 0.0005	0.817
Congestion index	0.14 ± 0.04	0.09 ± 0.03	< 0.0005	0.797
Portal hypertensive index	3.18 ± 0.90	2.14 ± 0.77	< 0.0005	0.791

LEV: Large 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.

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

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 LE 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 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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S- Editor Yang RH **L- Editor** Mihm S **E- Editor** Liu Y

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World J Gastroenterol 2008 March 28; 14(12): 1903-1907
World Journal of Gastroenterology ISSN 1007-9327

RAPID COMMUNICATION

Importance of the surrounding colonic mucosa in distinguishing between hyperplastic and adenomatous polyps during acetic acid chromoendoscopy

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Received: August 26, 2007 Revised: February 3, 2008

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.

Key words: Colon polyp; Chromoendoscopy; Acetic acid

Peer reviewers: Luis Rodrigo, Professor, Gastroenterology Service, Hospital Central de Asturias, c/ Celestino Villamil, s.n., Oviedo 33.006, Spain; Javier San Martin, Gastroenterology and Endoscopy, Sanatorio Cantegril, Av. Roosevelt y P 13, Punta del Este 20100, Uruguay

Kim JH, Lee SY, Kim BK, Choe WH, Kwon SY, Sung IK, Park HS, Jin CJ. Importance of the surrounding colonic mucosa in distinguishing between hyperplastic and adenomatous polyps during acetic acid chromoendoscopy. *World J Gastroenterol* 2008; 14(12): 1903-1907 Available from: URL: http://www.wjgnet.com/1007-9327/14/1903.asp DOI: http://dx.doi.org/10.3748/wjg.14.1903

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 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.

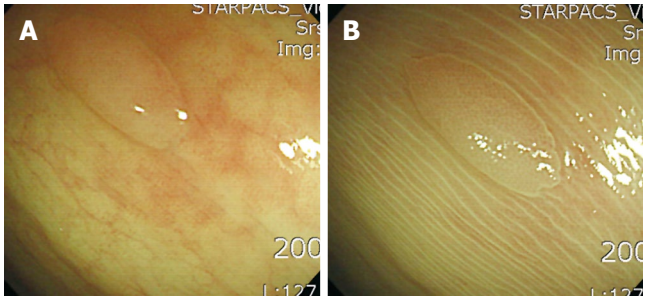

Figure 1 Linear and regularly patterned colonic mucosa surrounding a polyp. On endoscopic removal, pathology revealed a hyperplastic colon polyp. **A**: Before spraying with acetic acid a sessile polyp is seen; **B**: After spraying with acetic acid the colonic mucosa surrounding the polyp has a linear and regular pattern.

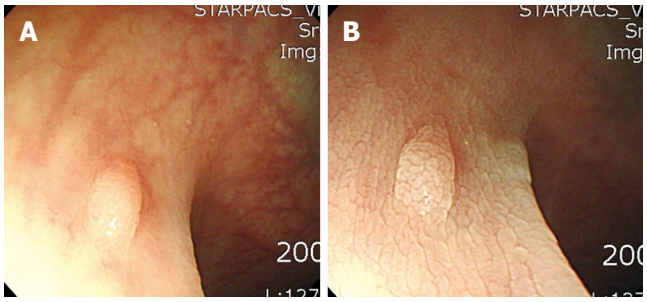

Figure 2 Nodular and irregularly patterned colonic mucosa surrounding a polyp. On endoscopic removal, pathology revealed a hyperplastic colon polyp. **A**: Before spraying with acetic acid a sessile polyp is seen; **B**: After spraying with acetic acid the colonic mucosa surrounding the polyp has a nodular and irregular pattern.

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 (*n* = 34)	Nodular and irregular (*n* = 16)	*P* 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.

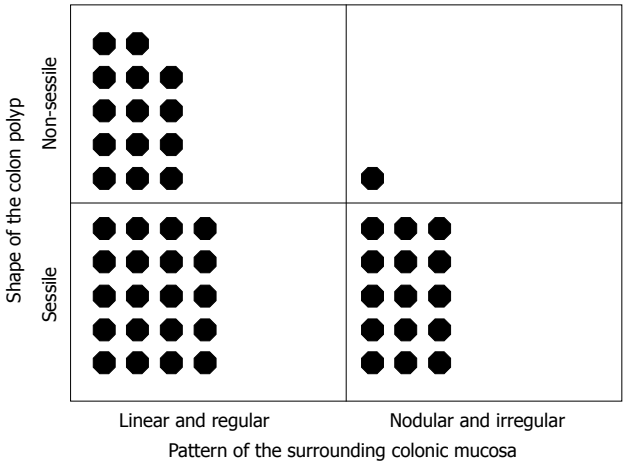

Figure 3 Diagnosis according to the shape of the colon polyp and the pattern of its surrounding colonic mucosa. Each black dot indicates a polyp. Most of the non-sessile colon polyps revealed linear and regular patterned surrounding colonic mucosa ($P = 0.02$).

(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 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 from16 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.

ACKNOWLEDGMENTS

The authors would like to thank to Professor Soo Nyung Kim for his assistance in statistical analysis.

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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S- Editor Denk H **L- Editor** Alpini GD **E- Editor** Ma WH

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World J Gastroenterol 2008 March 28; 14(12): 1908-1913
World Journal of Gastroenterology ISSN 1007-9327

RAPID COMMUNICATION

Hemodynamic effects of propranolol with spironolactone in patients with variceal bleeds: A randomized controlled trial

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Received: October 10, 2007 Revised: January 3, 2007

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 $\leqslant$ 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.

Key words: Hepatic venous pressure gradient; Secondary prophylaxis; Spironolactone; Propranolol; Variceal bleeding

Peer reviewer: Christer S von Holstein, Associate Professor, Department of Surgery, Lund University Hospital, SE-221 85 Lund, Sweden

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 $\leqslant$ 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 Ireland, 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, which ever came earlier. Hemodynamic studies were repeated on the eight 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 $\leqslant$ 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 ± 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 *n* = 17	Group B *n* = 18	*P* 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 hyper dynamic 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 HVPG 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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S- Editor Zhu WL **L- Editor** McGowan D **E- Editor** Liu Y

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World J Gastroenterol 2008 March 28; 14(12): 1914-1918
World Journal of Gastroenterology ISSN 1007-9327

RAPID COMMUNICATION

Effect of *H pylori* infection and its eradication on hyperammo-nemia and hepatic encephalopathy in cirrhotic patients

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Received: October 24, 2007 Revised: December 30, 2007

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. ^{14}C 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 μmol/L, respectively (P < 0.01), which was significantly reduced to 53.5 ± 37.7 μmol/L after bacterium eradication (n = 126) (P < 0.01). Blood ammonia was 97.5 ± 81.0 μmol/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* eradiation (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 μmol/L), SHE (59.9 ± 49.2 μmol/L), and without HE (47.3 ± 33.5 μmol/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.

Key words: Cirrhosis; *Helicobacter Pylori*; Hepatic encephalopathy; Hyperammonemia

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Chen SJ, Wang LJ, Zhu Q, Cai JT, Chen T, Si JM. Effect of *H pylori* infection and its eradication on hyperammonemia and hepatic encephalopathy in cirrhotic patients. *World J Gastroenterol* 2008; 14(13): 1914-1918 Available from: URL: http://www.wjgnet.com/1007-9327/14/1914.asp DOI: http://dx.doi.org/10.3748/wjg.14.1914

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 ^{14}C 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 (μmol/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

	H pylori (+)	*H pylori* (-)	*P* value
Sex (male/female)	196/81	141/39	0.045
Age (yr)	57.6 ± 12.7	56.9 ± 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 (omeparazole 20 mg b.i.d plus clarythromicin 500 mg b.i.d plus tinidazole 500 mg b.i.d). One month after completion of treatment, a ^{14}C-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 ± 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 ± 51.4 and 78.4 ± 63.6 μmol/L, respectively ($P < 0.01$). Blood ammonia was 78.4 ± 63.6 μmol/L in *H pylori*-positive cirrhotic patients before treatment ($n = 137$), and 97.5 ± 81.0 μmol/L in those with persistent infection after treatment ($n = 11$). Blood ammonia was significantly reduced to 53.5 ± 37.7 μmol/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* eradiation ($P < 0.01$). 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

H pylori infection	*n*	Ammonia concentration (μmol/L)	HE rate
H pylori (–)	180	53.8 ± 51.4[bd]	55 (30.6%)[bd]
H pylori (+)	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 (*n* = 217)	SHE (*n* = 55)	Cirrhotic (*n* = 62)	*P* value	χ^2
H pylori 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).

Table 4 Risk factors for HE analyzed by logistic multiple regression

	P 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
H pylori 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

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 ($P < 0.01$). 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 10^{10} 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* eradiation ($P < 0.01$). 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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S- Editor Liu JN **L- Editor** Kerr C **E- Editor** Ma WH

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World J Gastroenterol 2008 March 28; 14(12): 1919-1924
World Journal of Gastroenterology ISSN 1007-9327

RAPID COMMUNICATION

Changes of ghrelin following oral glucose tolerance test in obese children with insulin resistance

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Received: November 15, 2007 Revised: January 16, 2008

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.

Key words: Ghrelin; Oral glucose tolerance test; Insulin resistance; Obese children

Peer reviewers: Valerio Nobili, Dr, Liver Unit, Research Institute, Bambino Gesù Children's Hospital, S. Onofrio 4 Square, 00165 Rome, Italy; Laura E Matarese, MS, RD, LDN, FADA, CNSD, Thomas E. Starzl Transplantation Institute, UPMC Montefiore, 7 South, 3459 Fifth Avenue, Pittsburgh, PA 15213, United States

Wang XM, Jiang YJ, Liang L, Du LZ. Changes of ghrelin following oral glucose tolerance test in obese children with insulin resistance. *World J Gastroenterol* 2008; 14(12): 1919-1924 Available from: URL: http://www.wjgnet.com/1007-9327/14/1919.asp DOI: http://dx.doi.org/10.3748/wjg.14.1919

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/m^2. 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/m^2), 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 gherlin 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℃ 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 ^{125}I-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 ·1^{-2}				
Mean (LOG10)	-1.90 ± 0.38	-1.87 ± 0.24	-1.89 ± 0.51	-2.00 ± 0.10
HOMA-IR-mIU ·mmol ·1^{-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.

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[e]	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.

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 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
	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
BT-Ⅱ	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
	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
	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
	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 supression 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 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

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	0.488	0.35	0.297	0.552	0.435	0.737
	(0.015)[a]	(0.326)	(0.947)	(0.568)	(0.269)	(0.338)	(0.095)
BT-Ⅱ	0.045	0.1	0.929	0.896	0.772	0.85	0.672
	(0.859)	(0.693)	(0.007)[a]	(0.016)[a]	(0.072)	(0.032)[a]	(0.114)
GT- I	0.074	0.194	0.25	0.027	0.206	0.018	0.065
	(0.889)	(0.062)	(0.633)	(0.959)	(0.296)	(0.973)	(0.903)
GT-Ⅱ	0.135	0.551	0.668	0.466	0.419	0.301	0.557
	(0.829)	(0.336)	(0.218)	(0.299)	(0.482)	(0.622)	(0.330)

[a]$P < 0.05$.

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.

ACKNOWLEDGMENTS

We thank all the children and their parents for participating in this research project. We also thank You-Jun Jiang, Chun-Lin Wang, and Hong Shen for their exceptional patient care and organization.

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 in to 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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S- Editor Piscaglia AC **L- Editor** Alpini GD **E- Editor** Lu W

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World J Gastroenterol 2008 March 28; 14(12): 1925-1930
World Journal of Gastroenterology ISSN 1007-9327

RAPID COMMUNICATION

Comparative analysis of common *CFTR* polymorphisms poly-T, TG-repeats and M470V in a healthy Chinese population

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Supported by The Key Talent of Traditional Chinese Medicine, Foundation from the Government of Jiangsu Province, No. [2007] 158
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Received: September 14, 2007 Revised: December 21 , 2007

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.

Key words: Cystic fibrosis transmembrane conductance regulator gene; Gene polymorphism; Poly-T; TG-repeats; M470V

Peer reviewer: CS Pitchumoni, Professor, Robert Wood Johnson School of Medicine, Robert Wood Johnson School of Medicine, New Brunswick, NJ D8903, United States

Huang Q, Ding W, Wei MX. Comparative analysis of common *CFTR* polymorphisms poly-T, TG-repeats and M470V in a healthy Chinese population. *World J Gastroenterol* 2008; 14(12): 1925-1930 Available from: URL: http://www.wjgnet.com/1007-9327/14/1925.asp DOI: http://dx.doi.org/10.3748/wjg.14.1925

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 100 000, and in particular, the severe mutations, such as ΔF508, 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 Jingsu 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℃.

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℃ for 5 min for preheating, 35 cycles at 94℃ for 30 s, 60℃ for 60 s, and 72℃ for 30 s, followed by one cycle at 72℃ for 10 min for extension. The oligonucleotide primers used were: Intron 8 and exon 9 junction, sense 5'-CCATGTGCTTTTCAAACTAAT 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 *Hph*I restriction enzyme digestion.

Haplotype analysis

Haplotypes consisting of three loci were investigated, that is, poly-T, TG-repeats, and the M470V. The M470V polymorphisms were estimated by the AGE photos of pure PCR products after *Hph*I 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.

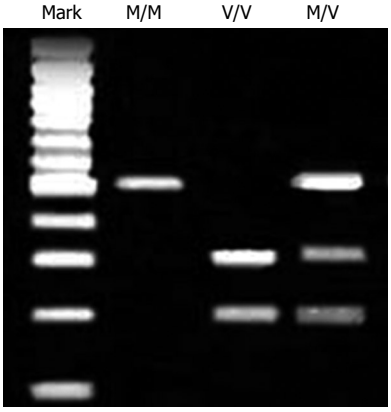

Figure 1 M470V polymorphism in exon 10.

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)

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).

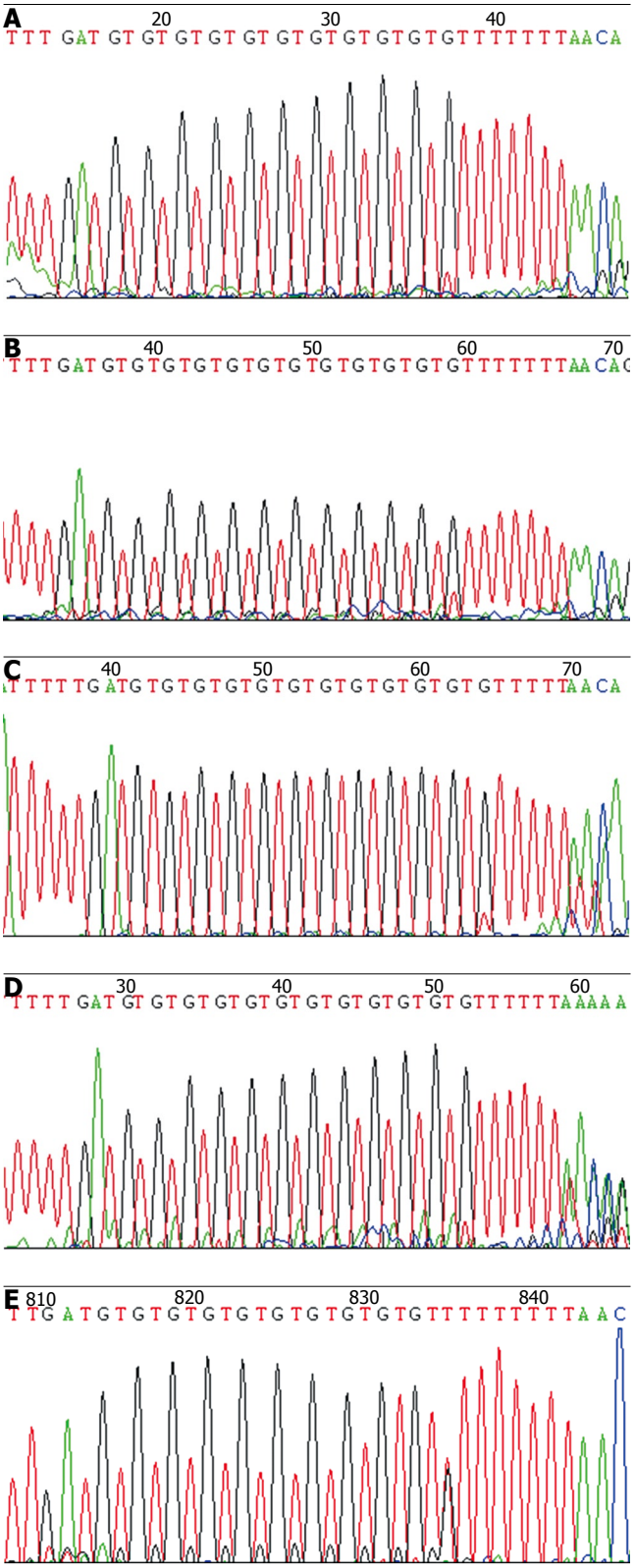

Figure 2 The polymorphisms of TG-repeats and Poly-T haplotype in IVS-8. **A**: TG11-T7; **B**: TG12-T7; **C**: TG12-T5; **D**: TG12-T6; **E**: TG10-T9.

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 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.

Table 2 Allele frequency of TG-repeat polymorphisms

Number ($2n$)	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)

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 bronch iectasis[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 ΔF508[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 CTFR 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.

ACKNOWLEDGMENTS

We thanks Dr. S Naruse (Department of Internal Medicine, Nagoya University, Nagoya, Japan) for his excellent technical guidance.

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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S- Editor Yang RH **L- Editor** Kerr C **E- Editor** Ma WH

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World J Gastroenterol 2008 March 28; 14(12): 1931-1935
World Journal of Gastroenterology ISSN 1007-9327

RAPID COMMUNICATION

Prognostic significance of S100A4 and vascular endothelial growth factor expression in pancreatic cancer

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Received: October 25, 2007 Revised: January 1, 2008

Peer reviewer: Kazuhiro Hanazaki, MD, Professor and Chairman, Department of Surgery, Kochi Medical School, Kochi University, Kohasu, Okohcho, Nankoku, Kochi 783-8505, Japan

Ai KX, Lu LY, Huang XY, Chen W, Zhang HZ. Prognostic significance of S100A4 and vascular endothelial growth factor expression in pancreatic cancer. *World J Gastroenterol* 2008; 14(12): 1931-1935 Available from: URL: http://www.wjgnet.com/1007-9327/14/1931.asp DOI: http://dx.doi.org/10.3748/wjg.14.1931

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.

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 granulae 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%) 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).

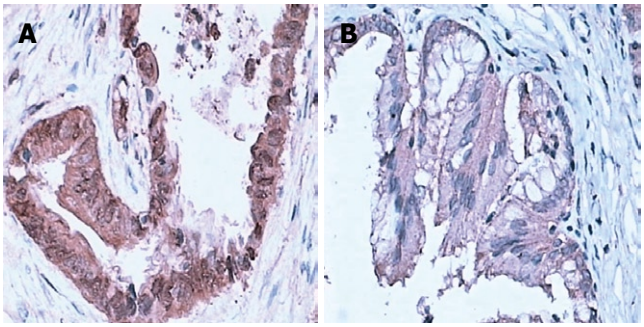

Figure 1 Positive expression of S100A4 (**A**) and VEGF (**B**) in pancreatic cancer (× 200).

Table 1 Correlation analysis of S100A4 and VEGF expression in pancreatic cancer

S100A4	VEGF		*P* value
	(+)	(-)	
(+)	30	8	
(-)	7	17	< 0.001

Significance was estimated with χ^2 test.

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 VFGF 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 (*n*)	S100A4 expression		*P* value
		Positive	Negative	
Age (yr)				
⩾ 70	24	15 (62.5)	9 (37.5)	
< 70	38	23 (60.5)	15 (39.5)	> 0.05[1]
Gender				
Male	36	22 (61.9)	14 (38.1)	
Female	26	16 (61.5)	10 (38.5)	> 0.05[1]
Differentiation				
Well	17	12 (70.6)	5 (29.4)	
Moderately	15	9 (60.0)	6 (40.0)	
Poorly	30	17 (56.7)	13 (43.3)	> 0.05[1]
Tumor size (cm)				
< 2.0	3	0 (0)	3 (100)	
2.0-4.0	38	21 (55.3)	17 (44.7)	
> 4.0	21	17 (81.0)	4 (19.0)	< 0.05[2]
Lymph node metastasis				
(-)	13	5 (38.5)	8 (61.5)	
(+)	49	33 (67.3)	16 (32.7)	> 0.05[1]
Distant metastasis				
(-)	39	23 (59.0)	16 (41.0)	
(+)	23	15 (65.2)	8 (34.8)	> 0.05[1]
TNM stage				
Ⅰ + Ⅱ	30	14 (46.7)	16 (53.3)	
Ⅲ + Ⅳ	32	24 (75.0)	8 (25.0)	< 0.05[1]

[1]Significance was estimated with χ^2 test; [2]Significance was estimated with Fisher's exact test.

Table 3 Correlation between VEGF expression and clinicopathological parameters in pancreatic cancer, *n* (%)

Clinicopathological parameters	Cases (*n*)	VEGF expression		*P* value
		Positive	Negative	
Age (yr)				
⩾ 70	24	17 (70.8)	7 (29.2)	
< 70	38	20 (52.6)	18 (47.4)	> 0.05
Gender				
Male	36	24 (66.7)	12 (33.3)	
Female	26	13 (50)	13 (50)	> 0.05
Differentiation				
Well	17	10 (58.8)	7 (41.2)	
Moderately	15	8 (53.3)	7 (46.7)	
Poorly	30	19 (63.3)	11 (36.7)	> 0.05
Tumor size (cm)				
< 2.0	3	2 (66.7)	1 (33.3)	
2.0-4.0	38	21 (55.3)	17 (44.7)	
> 4.0	21	14 (66.7)	7 (33.3)	> 0.05
Lymph node metastasis				
(-)	13	7 (53.8)	6 (46.2)	
(+)	49	30 (61.2)	19 (38.8)	> 0.05
Distant metastasis				
(-)	39	24 (61.5)	15 (38.5)	
(+)	23	13 (56.5)	10 (43.5)	> 0.05
TNM stage				
Ⅰ + Ⅱ	30	17 (56.7)	13 (43.3)	
Ⅲ + Ⅳ	32	20 (62.5)	12 (37.5)	> 0.05

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 significant difference between the S100A4(+)/VEGF(-) and S100A4(+)/VEGF(-) groups (Figure 2C).

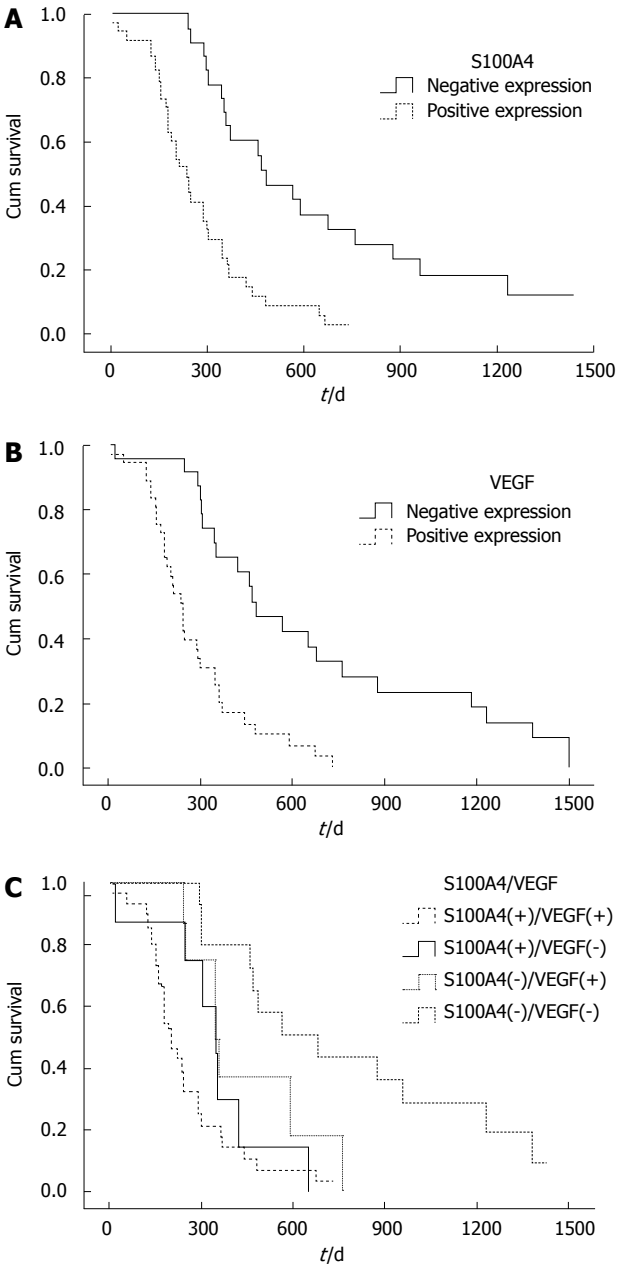

Figure 2 Survival curves for group of pancreatic cancer patients according to S100A4 expression (**A**), group of pancreatic cancer patients according to VEGF expression (**B**), and four subgroups of pancreatic cancer patients according to the expression of S100A4 and VEGF (**C**).

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 and 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 secret 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 21members, 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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World J Gastroenterol 2008 March 28; 14(12): 1936-1940
World Journal of Gastroenterology ISSN 1007-9327

RAPID COMMUNICATION

Double-balloon enteroscopy reliably directs surgical intervention for patients with small intestinal bleeding

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Received: April 4, 2007 Revised: December 24, 2007

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.

Key words: Double-balloon enteroscopy; Small intestine bleeding; Surgery

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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

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 from14-75 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	
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 120º 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 patients, the retrograde approach in 2 patients, and both in 4 patients.

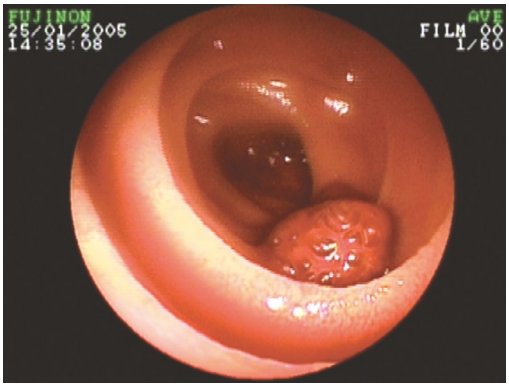

Figure 1 Enteroscopy identified a polyp 1.5 cm in diameter in jejunum.

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

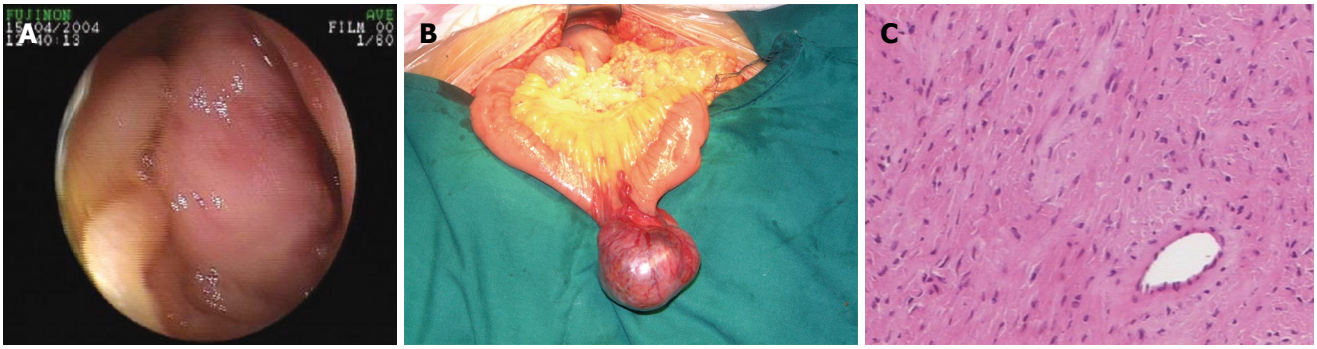

Figure 2 Stromal tumor of small intestine. **A**: Preoperative enteroscopy; **B**: Surgical exploration; **C**: Photomicrograph of the lesion (HE, × 100).

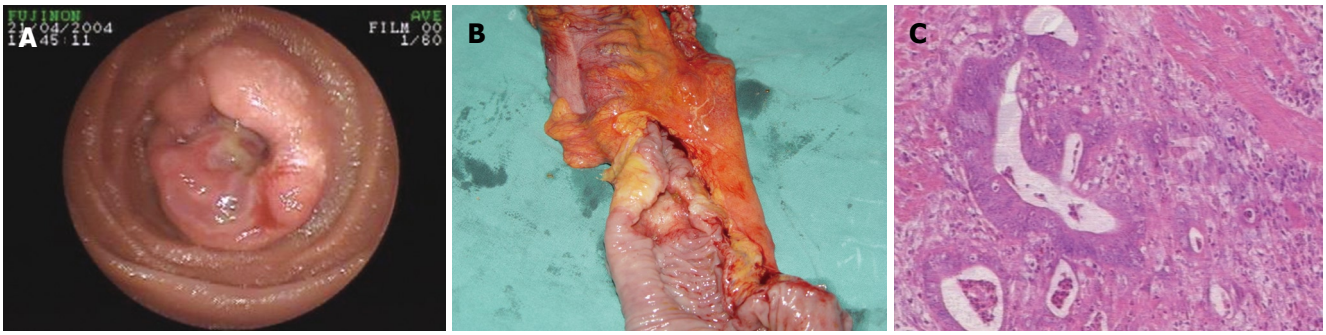

Figure 3 Adenocarcinoma of small intestine. **A**: Preoperative enteroscopy; **B**: Surgical exploration; **C**: Photomicrograph of the lesion (HE, × 100).

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 malignances 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]. GITS 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 enteroscope is limited in its mucosal visualization and can only detect the lump within the bowel lumen. Therefore, preoperative enteroscopy has little value in predicting

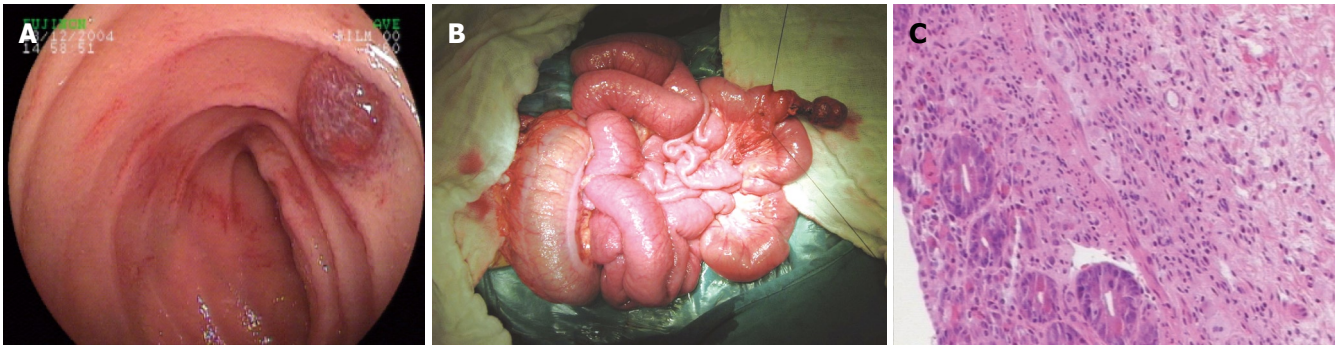

Figure 4 Diverticula was visualized as angeioma by preoperative double-balloon enteroscopy. **A**: Preoperative enteroscopy, angeioma between jejunum and ileum; **B**: Diverticula 80 cm proximal to ileocecum. **C**: Photomicrograph of the lesion (HE, × 100).

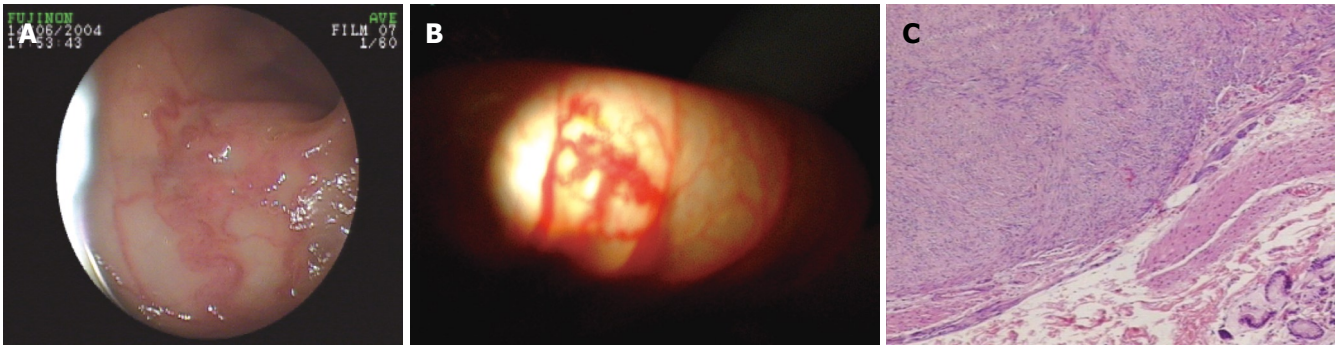

Figure 5 Vascular malformation of small intestine. **A**: Preoperative enteroscopy; **B**: Surgical exploration; **C**: Photomicrograph of the lesion (HE, × 100).

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 (Dieulafory'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 anterograde 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 endocopy 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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World J Gastroenterol 2008 March 28; 14(12): 1941-1945
World Journal of Gastroenterology ISSN 1007-9327

RAPID COMMUNICATION

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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Author contributions: Tao XH, Gao Q, and Liang XY designed the study and wrote the paper; Liang XY, Gong NP, and Tang LP collected and analyzed the data; Wang PL and Tao XH provided valuable guidance during the study.

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Received: October 6, 2007 Revised: December 4, 2007

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.

Key words: Esomeprazole; Duodenal ulcer; Proton pump inhibitor

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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

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.

Table 1 Baseline demographic and clinical characteristics (%, mean ± 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)	χ^2 = 0.3725	0.5416
Age (yr)	44.2 ± 11.9	43.6 ± 11.1	t = -0.1981	0.8437
Height (cm)	163.1 ± 7.2	164.7 ± 7.0	t = 0.8339	0.4079
Systolic BP (mmHg)	114.5 ± 10.5	111.6 ± 8.2	t = -1.1730	0.2459
Diastolic BP (mmHg)	73.4 ± 7.4	74.3 ± 9.2	t = 0.3901	0.6980
Heart rate (bpm)	73.8 ± 8.5	75.1 ± 11.3	t = 0.4713	0.6393
Duration of DU (mo)	58.3 ± 61.2	58.6 ± 53.7	t = 0.0178	0.9859
Total score of symptoms	5.5 ± 2.6	5.2 ± 1.6	t = -0.5580	0.5794
Ulcer diameter (mm)	7.7 ± 2.6	7.5 ± 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)	χ^2 = 0.6621	0.4158

SD: Standard deviation; BPM: Beats per minute; DU: Duodenal ulcer.

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, 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)] × 100%.

Assessment of adverse events

The safety and tolerability of the medication were assessed using physical examination at final visit, review

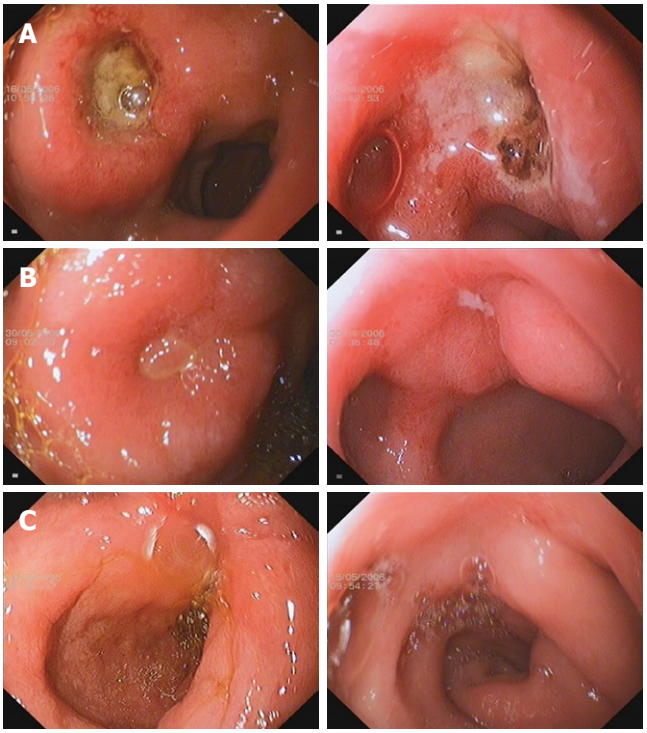

Figure 1 Comparison of Esomeprazole enteric-coated capsules treated group (left column) and Esomeprazole magnesium treated group (right column) in duodenal ulcer under endoscopy. **A**: Baseline duodenal ulcer under endoscopy; **B**: Duodenal ulcer under endoscopy at wk 2; **C**: Duodenal ulcer under 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 $\leqslant 0.05$. 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 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.

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

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 improv-ing 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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S- Editor Ma L **L- Editor** McGowan D **E- Editor** Liu Y

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World J Gastroenterol 2008 March 28; 14(12): 1946-1948
World Journal of Gastroenterology ISSN 1007-9327

CASE REPORT

Investigation of the excluded stomach after Roux-en-Y gastric bypass: The role of percutaneous endoscopy

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Received: September 19, 2007 Revised: December 7, 2007

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.

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 approaches 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 transabdominal 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

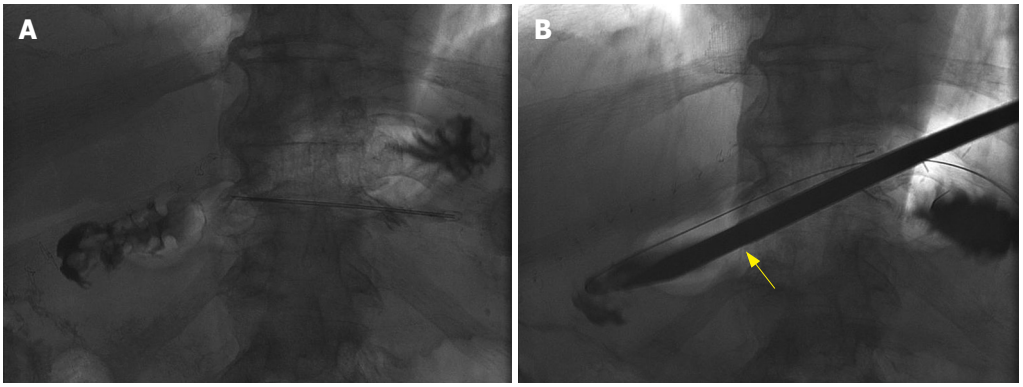

Figure 1 Fluoroscopic images of percutaneous endoscopy. **A**: Inflated bypassed stomach with air; **B**: Trochar (arrow) used to dilate the percutaneous track.

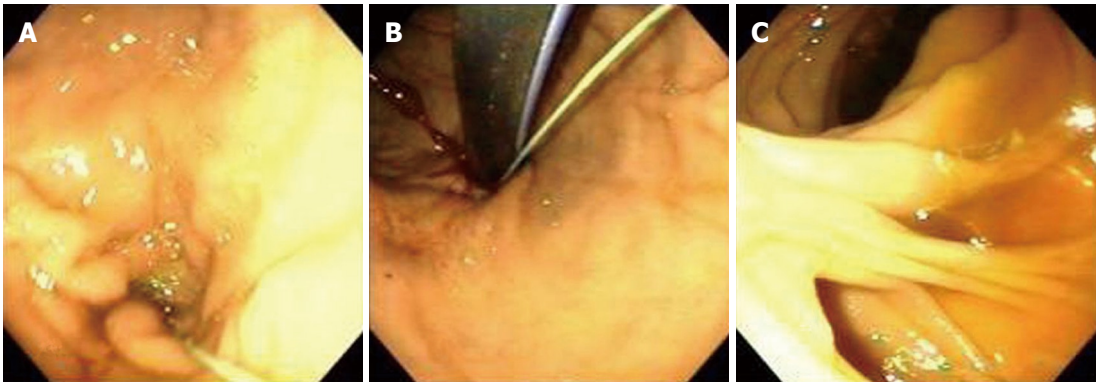

Figure 2 Endoscopic images by percutaneous endoscopy. **A**: Antegrade views of bypassed stomach; **B**: Retrograde views of bypassed stomach; **C**: Roux-en-Y anastomosis.

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.

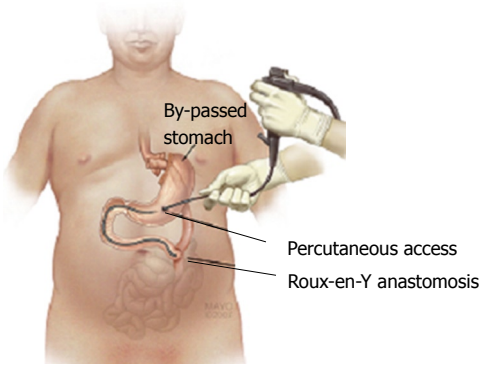

Figure 3 Illustration of the percutaneous endoscopy.

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 common 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 BDE 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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World J Gastroenterol 2008 March 28; 14(12): 1949-1951
World Journal of Gastroenterology ISSN 1007-9327

CASE REPORT

Conservative management of perforated duodenal diverticulum: A case report and review of the literature

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Received: July 19, 2007 Revised: January 10, 2008

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.

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/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 retropneumoperitoneum 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 nill-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 subdiphragmatic, 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,

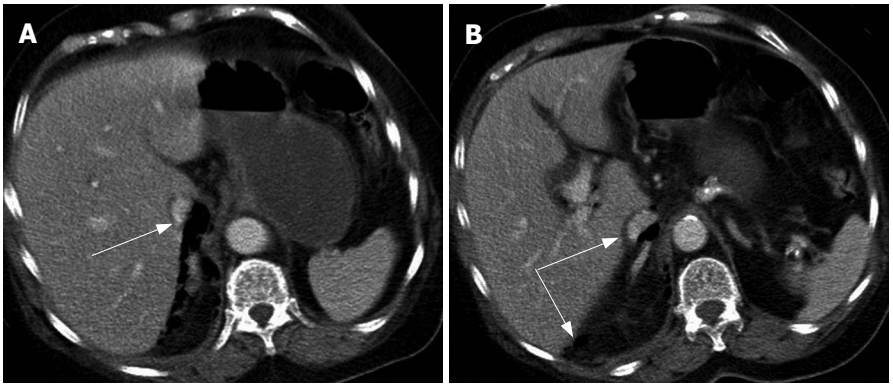

Figure 1 Abdominal CT showing perihepatic free liquid, pneumoperitoneum and retropneumoperitoneum next to the cava vein.

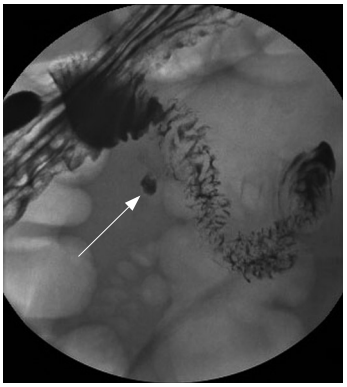

Figure 2 Gastrografin swallow examination revealing a duodenal diverticulum in the second portion without leakage of contrast.

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 pneumony.

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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World J Gastroenterol 2008 March 28; 14(12): 1952-1954
World Journal of Gastroenterology ISSN 1007-9327

CASE REPORT

Paraneoplastic hyperinsulinism and secondary hypoglycaemia in a patient with advanced colon cancer: A rare association

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Received: January 1, 2007 Revised: January 25, 2008

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.

Key words: Hypoglycemia; Colon cancer; Paraneoplastic; Hyperinsulinism; Tumour markers

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Diaz R, Aparicio J, Mendizabal A, Faus M, Fleitas T, Aparisi F, Martin M. Paraneoplastic hyperinsulinism and secondary hypoglycaemia in a patient with advanced colon cancer: A rare association. *World J Gastroenterol* 2008; 14(12): 1952-1954 Available from: URL: http://www.wjgnet.com/1007-9327/14/1952.asp DOI: http://dx.doi.org/10.3748/wjg.14.1952

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 μUI/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 octeotride 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 paraneoplasic 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 multiorganic 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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World J Gastroenterol 2008 March 28; 14(12): 1955-1957
World Journal of Gastroenterology ISSN 1007-9327

CASE REPORT

Extraction and clipping repair of a chicken bone penetrating the gastric wall

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Received: September 20, 2007 Revised: December 10, 2007

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, endoclipping can be a useful method in patients with no signs or symptoms of peritoneal irritation.

Key words: Gastric penetration; Chicken bone; Hemoclip

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Kim JS, Kim HK, Cho YS, Chae HS, Kim CW, Kim BW, Han SW, Choi KY. Extraction and clipping repair of a chicken bone penetrating the gastric wall. *World J Gastroenterol* 2008; 14(12): 1955-1957 Available from: URL: http://www.wjgnet.com/1007-9327/14/1955.asp DOI: http://dx.doi.org/10.3748/wjg.14.1955

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℃. The patient had an acute ill appearance but no history of nonsteroidal anti-inflammatory drug use, peptic ulcer, or liver disease. Laboratory studies

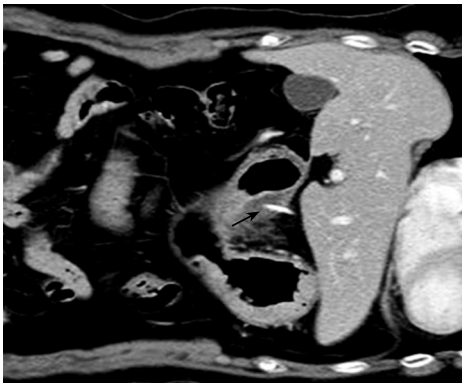

Figure 1 2.8 cm linear density (arrow) penetrating into the thickened gastric wall (CT).

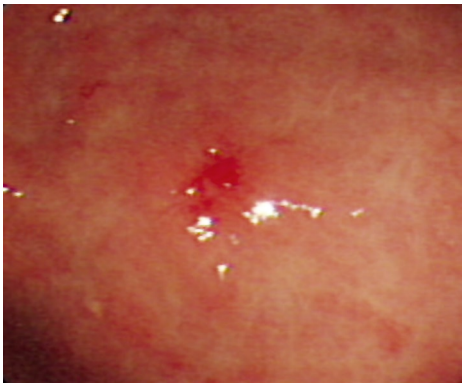

Figure 3 Follow-up endoscopy revealing near closure of the ulcer at the penetration site.

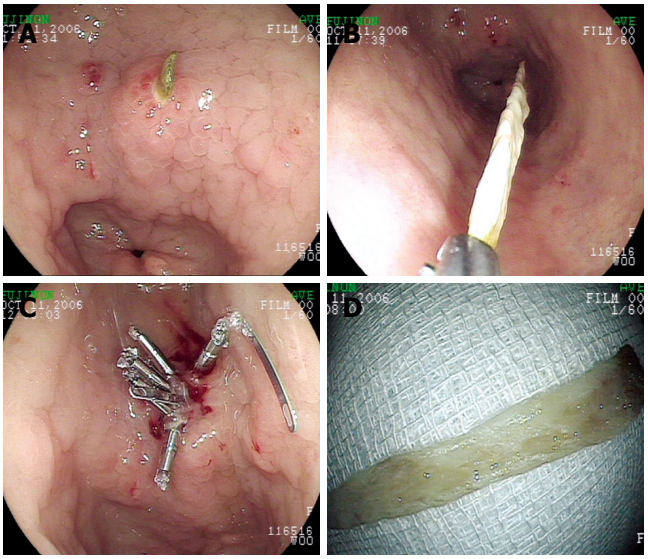

Figure 2 Endoscopic management. **A**: A chicken bone penetrating into the prepyloric antrum; **B**: The whole chicken bone was removed using a grasping forcep; **C**: Clip placement was performed at the removal site; **D**: The length of removed chicken bone was about 3.0 cm.

revealed hemoglobin concentration of 132 g/L, hematocrit 38.6%, white blood cell count of 11.7 × 10^9/L, and platelet count of 222 × 10^9/L. Her serum urea nitrogen concentration was 3.2 mmol/L, creatinine 50.4 μmol/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 bonegragment, 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, endoclipping 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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S- Editor Ma L **L- Editor** Wang XL **E- Editor** Ma WH

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World J Gastroenterol 2008 March 28; 14(12): 1958-1960
World Journal of Gastroenterology ISSN 1007-9327

CASE REPORT

Growth process of small pancreatic carcinoma: A case report with imaging observation for 22 months

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Received: September 20, 2007 Revised: December 8, 2007

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.

Key words: Invasive ductal carcinoma; Pancreas; Intraductal papillary-mucinous neoplasm; Endoscopic ultrasonography; Tumor volume doubling time

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Hisa T, Ohkubo H, Shiozawa S, Ishigame H, Takamatsu M, Furutake M, Nobukawa B, Suda K. Growth process of small pancreatic carcinoma: A case report with imaging observation for 22 months. *World J Gastroenterol* 2008; 14(12): 1958-1960 Available from: URL: http://www.wjgnet.com/1007-9327/14/1958.asp DOI: http://dx.doi.org/10.3748/wjg.14.1958

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.

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

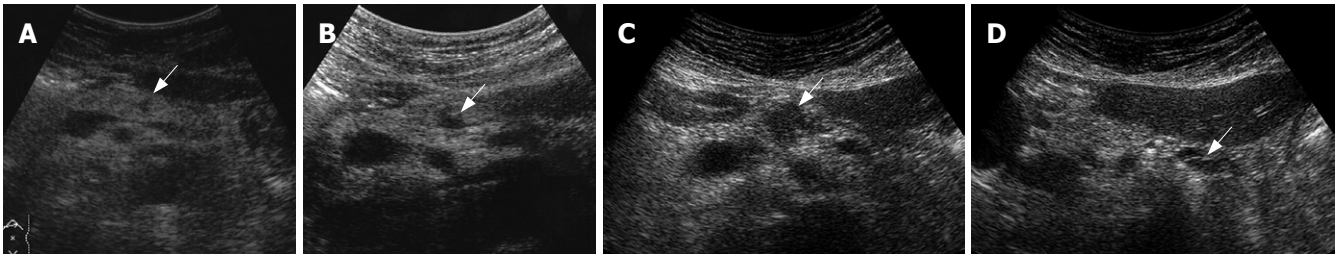

Figure 1 Chronological changes in US findings. **A**: Initial US showed a low-echoic mass, 7 mm in diameter, in the pancreatic body (arrow); **B**: After 10 mo, the diameter of the low-echoic mass had increased to 9 mm (arrow); **C**: After 22 mo, the diameter of the low-echoic mass had increased to 13 mm (arrow); **D**: After 22 mo, a grape-like cyst distal to the low-echoic mass was detected for the first time (arrow).

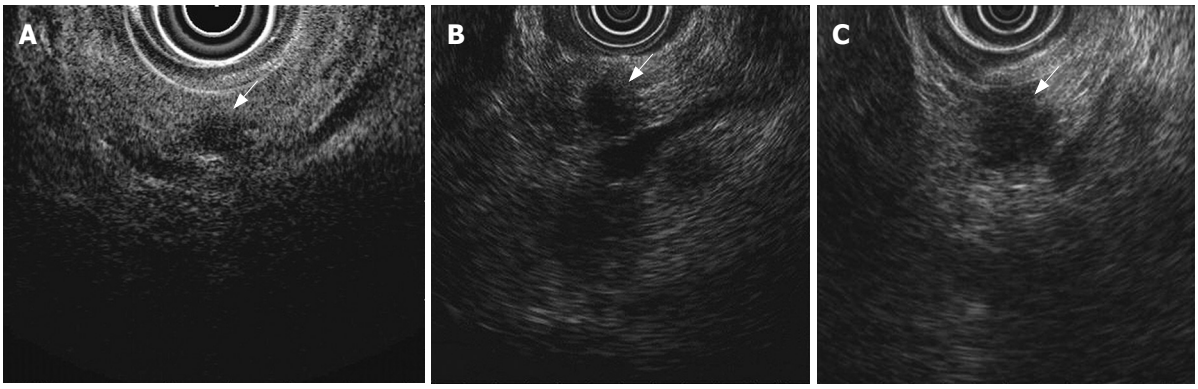

Figure 2 Chronological changes in EUS findings. **A**: Initial EUS demonstrated an extremely low-echoic mass with partial posterior echo enhancement, 7 mm in diameter, in the pancreatic body (arrow); **B**: After 14 mo, the diameter of the low-echoic mass had increased to 9 mm (arrow); **C**: After 22 mo, the diameter of the low-echoic mass had increased to 13 mm (arrow).

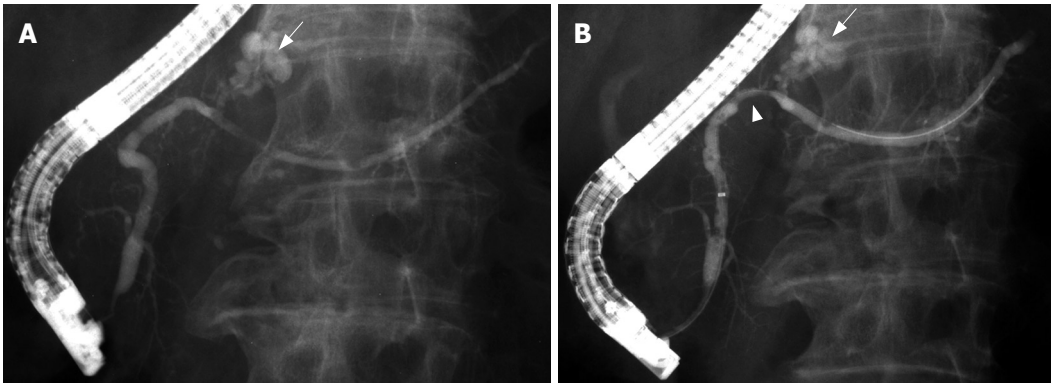

Figure 3 Chronological changes in ERP findings. **A**: Initial ERP revealed a dilated branch duct in the pancreatic body (arrow), and the MPD was mildly dilated by mucus; **B**: After 22 mo, the dilated branch duct did not show any marked change (arrow). Proximal to it, the MPD was slightly compressed (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-

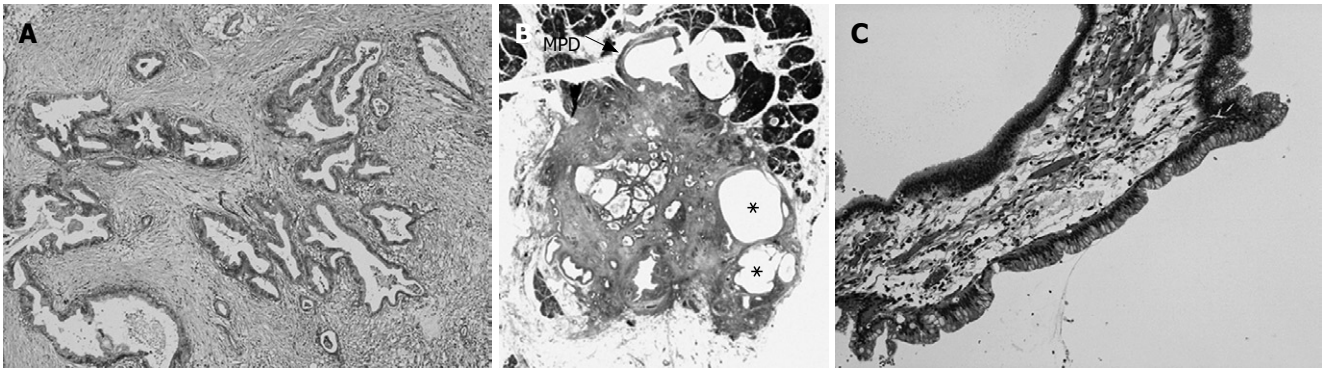

Figure 4 Histopathological findings. **A**: The mass is composed of moderately differentiated tubular adenocarcinoma with desmoplastic fibrosis, resulting in a diagnosis of IDC (HE, original magnification × 20); **B**: The tumor mass includes macroscopic cystic components (*) lined by normal ductal epithelium, suggestive of retention cysts in carcinoma (HE, original magnification × 1); **C**: The lining of the dilated branch duct is composed of low-papillary columnar cells with copious intracellular mucus, resulting in a diagnosis of branch duct type intraductal papillary-mucinous adenoma with mild atypia (HE, × 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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World J Gastroenterol 2008 March 28; 14(12): 1961-1963
World Journal of Gastroenterology ISSN 1007-9327

CASE REPORT

Peliosis and gummatous syphilis of the liver: A case report

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Received: October 15, 2007 Revised: January 10, 2008

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.

Key words: Focal liver lesion; Peliosis hepatis; Syphilis; Computed tomography

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Chen JF, Chen WX, Zhang HY, Zhang WY. Peliosis and gummatous syphilis of the liver: A case report. *World J Gastroenterol* 2008; 14(12): 1961-1963 Available from: URL: http://www.wjgnet.com/1007-9327/14/1961.asp DOI: http://dx.doi.org/10.3748/wjg.14.1961

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

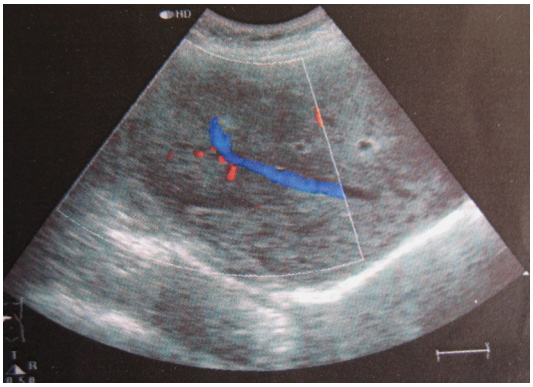

Figure 1 Doppler sonographic image showing a slightly heterogeneous hyperechoic lesion in the right lobe of liver without mass effect on the right hepatic vein.

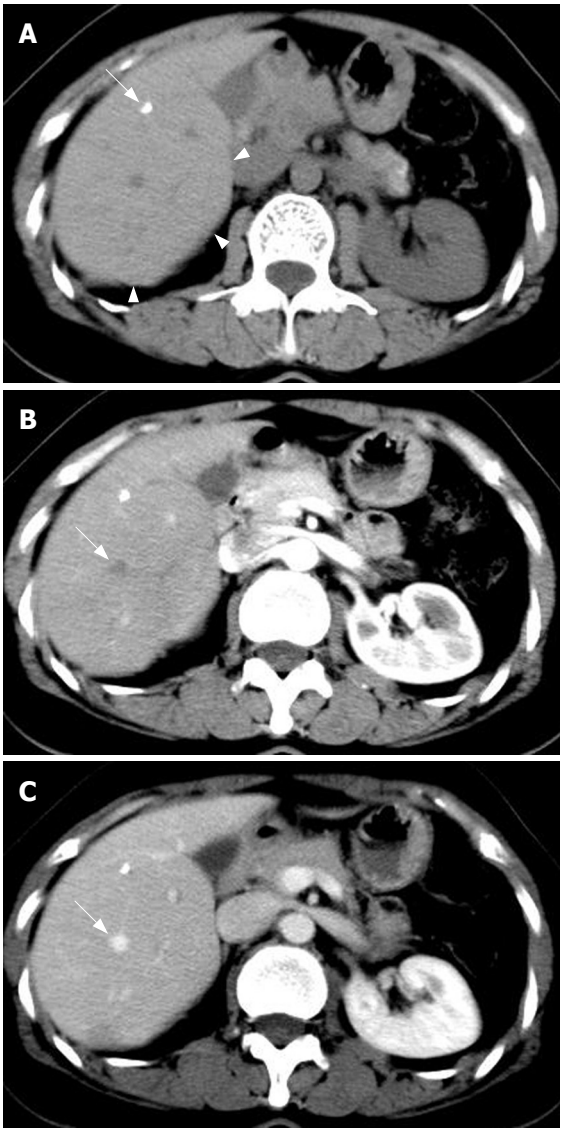

Figure 2 Transverse unenhanced CT image showing the protrudent visceral surface which hints a local iso-attenuated lesion (arrowheads) with punctate calcification (arrow) (**A**), identical density of the lesion to the adjacent liver parenchyma at arterial phase (**B**) and portal phase (**C**). The right hepatic vein with a normal shape and location crosses the lesion (arrow).

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,

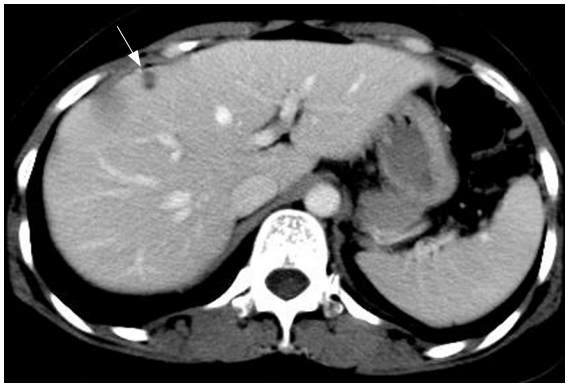

Figure 3 Portal phase image showing a hypo-attenuated lesion (10 mm in diameter) within segment Ⅷ (arrow). Histopathology proved it to be a gumma.

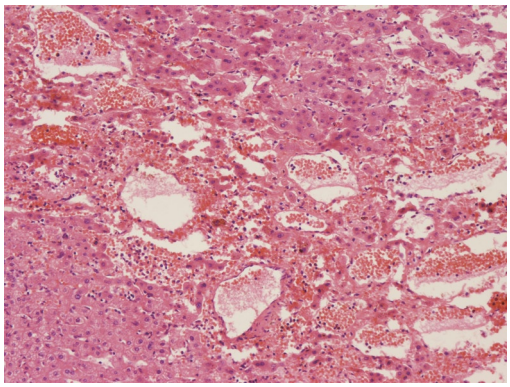

Figure 4 Histology of specimens (HE, × 50). Microscopy reveals multiple blood-filled cystic spaces and ectatic sinusoids.

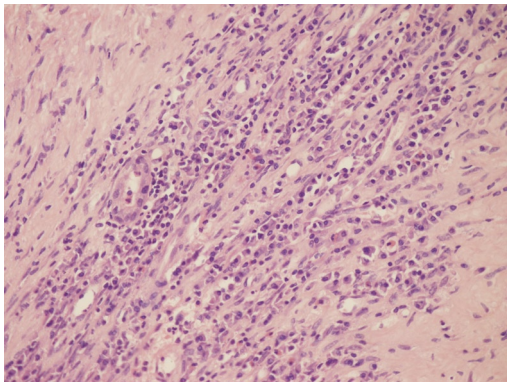

Figure 5 Gumma on segment Ⅷ characterized by multifocal coagulation necrosis and significant infiltration of plasma cells mixed with lymph cells (HE, × 100).

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.

ACKNOWLEDGMENTS

The authors thank Dr. Zhu HB for her valuable advice regarding the pathology of peliosis and gummatous syphilis of the liver and Dr. Song QL for help in the preparation of this manuscript.

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World J Gastroenterol 2008 March 28; 14(12): 1964
World Journal of Gastroenterology ISSN 1007-9327

ACKNOWLEDGMENTS

Acknowledgments to Reviewers of *World Journal of Gastroenterology*

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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Events Calendar 2008-2009

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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.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. B ile Acid Biology and Therapeutic Actions

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10th World Congress on Gastrointestinal Cancer
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June 26-28, Bratislava, Slovakia
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III FALK GASTRO-CONFERENCE
September 17, Mainz, Germany
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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
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October 8-11, Istanbul, Turkey
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www.negf.org
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59th AASLD Annual Meeting and Postgraduate Course
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Information: www.aasld.org

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Neurogastroenterology & Motility Joint International Meeting 2008
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6 21st century heart solution may have a sting in the tail. *BMJ*

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

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Chapter in a book (list all authors)

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

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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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Write as mean ± SD or mean ± SE.

Statistical expression

Express *t* test as *t* (in italics), *F* test as *F* (in italics), chi square test as χ^2 (in Greek), related coefficient as *r* (in italics), degree of freedom as υ (in Greek), sample number as *n* (in italics), and probability as *P* (in italics).

Units

Use SI units. For example: body mass, m (B) = 78 kg; blood pressure, p (B) = 16.2/12.3 kPa; incubation time, t (incubation) = 96 h, blood glucose concentration, c (glucose) 6.4 ± 2.1 mmol/L; blood CEA mass concentration, p (CEA) = 8.6 24.5 μg/L; CO_2 volume fraction, 50 mL/L CO_2, not 5% CO_2; likewise for 40 g/L formaldehyde, not 10% formalin; and mass fraction, 8 ng/g, *etc.* Arabic numerals such as 23, 243, 641 should be read 23 243 641.

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Italics

Quantities: *t* time or temperature, *c* concentration, *A* area, *l* length, *m* mass, *V* volume.
Genotypes: *gyrA*, *arg* 1, *c myc*, *c fos*, *etc.*
Restriction enzymes: *Eco*RI, *Hin*dI, *Bam*HI, *Kbo* I, *Kpn* I, *etc.*
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