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• *REVIEW* •

Immunological treatment of liver tumors

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Abstract

Although multiple options for the treatment of liver tumors have often been described in the past, including liver resection, radiofrequency ablation with or without hepatic pump insertion, laparoscopic liver resection and the use of chemotherapy, the potential of immunotherapy and gene manipulation is still largely unexplored. Immunological therapy by gene manipulation is based on the interaction between virus-based gene delivery systems and dendritic cells. Using viruses as vectors, it is possible to transduce dendritic cells with genes encoding tumor-associated antigens, thus inducing strong humoral and cellular immunity against the antigens themselves. Both chemotherapy and radiation therapy have the disadvantage of destroying healthy cells, thus causing severe side-effects. We need more precisely targeted therapies capable of killing cancer cells while sparing healthy cells. Our goal is to establish a new treatment for solid liver tumors based on the concept of cytoreduction, and propose an innovative algorithm.

Key words: Liver; Tumors; Surgery; Dendritic cell; Cytoreduction; Immunotherapy; Gene manipulation

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HEPATIC TUMORS

Although relatively uncommon in Western countries, hepatocellular carcinoma (HCC) is probably the most common solid cancer in the world, with an estimated incidence of at least one million new patients per year[1,2]. The optimal treatment for HCC is surgical excision with a curative intent, but only 5-15% of newly diagnosed patients undergo potentially curative resection[3]. Patients with disease confined to the liver may not be candidates for resection because of multifocal disease, or an inadequate hepatic functional reserve capacity related to co-existent cirrhosis may contraindicate resection. As there are few other curative treatment options for patients with unresectable liver disease, HCC is one of the most lethal human malignancies, with a mortality rate of 94%[4].

The liver is second only to lymph nodes as a site of metastases from other solid cancers[5], and may be the only site of metastatic disease particularly in patients with colorectal adenocarcinoma[6]. However, fewer than 10-15% of patients with liver metastases are candidates for resection for the same reasons as those regarding HCC. The majority of patients with primary or metastatic hepatic malignancies who are not candidates for complete surgical resection therefore require novel treatment modalities to control and potentially cure their disease[2,7].

Cirrhosis may be another variable that places such patients at the highest risk[2]. Patients in class C of the Child-Pugh Classification (Table 1) have the highest mortality and morbidity rate following all treatments, particularly surgical procedures[8,9], and so most centers have shifted away from open liver surgery and are attempting other approaches. The treatment of hepatic tumors in cirrhotic and non-cirrhotic patients is a major decision-making issue for oncologists and surgeons, and the high mortality rate of open liver surgery in cirrhotic patients has spurred physicians to seek new modalities[10].

We here outline the immunological and genetic techniques available for the treatment of liver tumors, and propose a new immunologico-clinical algorithm using immunological therapy to debulk the mass, kill micrometastases, and allow a lower dose of chemotherapy to achieve better cytoreduction.

Table 1 Child-Pugh classification

	A	B	C
Ascites	None	Controlled	Uncontrolled
Bilirubin (mmol/L)	<2.0	2.0-2.5	>3.0
Encephalopathy	None	Minimal	Advanced
PT (s prolonged)	<4.0	4.0-6.0	>6.0
INR	<2.0	2.0-3.0	>3
Albumin (g/L)	>3.5	3.0-3.5	<3.0

WHAT IS THE ROLE OF SURGERY?

Open surgery

Complete surgical resection of primary or secondary liver tumors is the gold standard of surgical therapy[2,8,9], but it has fallen out of favor because of complications related to bleeding and liver failure. Furthermore, the time of the associated hospitalization is not cost-effective in the context of the new health plan insurance capitation systems.

Underlying anatomical and physiological limitations may exclude the use of complete surgical resection but, when complete or partial resection is plausible, the approach of choice is either the traditional open technique (wedge resection, segmentectomy or major lobectomy) or the laparoscopic technique. Laparoscopic liver surgery has become feasible with the improvement in laparoscopic techniques and the development of new and dedicated technologies[9]. There are benefits common to all endoscopic procedures, and the choice of the approach to hepatic resection is usually made by both the surgeon and the patient.

Laparoscopic surgery

The laparoscopic method is useful in oncological therapy, as it allows abdominal exploration and the visualization of the tumor itself. Specimen collection is another key benefit, and can range from a lymph node biopsy in the peritoneum or retro-peritoneum, to scraping the peritoneum in the abdominal wall. Laparoscopy allows direct visualization of the organs and biopsy. The liver is a large organ, and can therefore be visualized quite well, particularly the anterior section, although it is laparoscopically more difficult to visualize the posterior section of the retroperitoneal area of the right lobe. Anatomically, the left side of the liver is not hard to mobilize by dissecting the left triangular ligament and flipping the left side of the liver over the midline, but it is more complicated to achieve the same result on the right side where segments VI and VII (the lateral segments) and segment VIII are harder to visualize posteriorly, and so intraoperative ultrasound has been introduced to improve the visualization of tumors in these segments[9].

Radiofrequency ablation

This is a thermal technique designed to cause localized tumor destruction by heating the tumoral tissue to temperatures of more than 50 °C. The methodology has been previously described by our group[8,11], and has been found to be safe and effective in the treatment of single tumors of <5 cm with curative intent, or the cytoreduction of multiple or larger tumors.

Percutaneous ethanol injection (PEI)

This is usually performed under transabdominal ultrasonographic guidance, and consists of intra-tumorally injecting 5-10 mL of ethanol twice a week. Patient compliance has been a problem because of the number of injections required and the associated pain. As PEI requires multiple treatment sessions and is associated with a high local recurrence rate, it should only be considered in the case of tumors with a diameter of less than 1.5 cm.

Cryosurgery

This has been used to treat patients with unresectable primary and metastatic liver tumors for the last 20 years. Most of the scientific data concerning local tumor recurrences and complications after cryosurgery comes from patients treated for colorectal cancer liver metastases[8].

CLINICAL ALGORITHM FOR SOLID LIVER TUMORS

The pros and cons of liver surgery and the new clinical algorithm used for the treatment of liver tumors will be briefly discussed[8], considering only the patients with Child –Pugh class A or B cirrhosis, because those with advanced liver cirrhosis (Child–Pugh class C) would probably receive no survival benefit and would be at a disproportionately increased risk of interventional therapy. The patients in the two groups will belong to one of the following four categories: (1) Those with stage I, primary liver tumors will be evaluated for liver resection or radiofrequency ablation (RFA); (2) Those with stage II and III primary liver tumors will undergo complete resection, if anatomically possible, or partial resection with RFA, or RFA alone; the patients with vascular invasion will also receive a hepatic arterial pump (HAP); (3) The patients with stage IV primary liver tumors or liver metastases of other than colorectal origin (endocrine, breast) will only be treated with RFA and a HAP; (4) The patients with colorectal metastases will undergo complete resection if possible, or partial resection with RFA, or RFA alone, and all will receive a HAP.

After a median follow-up of 20 mo in patients with unresectable liver disease, the addition of adjuvant HAP therapy to cryoreduction decreased all recurrences from 77% to 49% and decreased liver recurrences from 67% to 38%. This, and other multi-approaches (RFA and HAP therapy) to the treatment of partially resectable or unresectable liver disease, is promising and deserves further investigation.

IMMUNOTHERAPY AND NEOPLASTIC LIVER DISEASE

Most cancer patients are currently treated with some combination of surgery, radiation therapy and

chemotherapy, but both chemo- and radiation-therapy have the disadvantage of destroying healthy cells and this causes severe side effects. The possibility of destroying more cancer cells by increasing the chemotherapeutic dose or radiation exposure is limited by the non-specific organ toxicity of these therapies and the relatively old age of most patients. We therefore need more precisely targeted therapies capable of killing cancer cells while sparing healthy cells.

One possible answer is immunological therapy, which is not only more specific and less toxic, but may also induce memory responses that could yield long-term tumor immunosurveillance and reduce the incidence of relapses, thus increasing long-term disease-free survival. Immunological therapy may be adoptive[10,12] in which case the patients' white blood cells are coupled with a naturally producing growth factor to enhance their cancer fighting capacity, or passive[13], with immunity being acquired as a result of the transfer of antibodies from a healthy donor. However, the possibility of successfully implementing these therapies rests on the existence of tumor-specific antigens, and suitable antigens have been hard to come by because of the complex process required to validate them[14–18].

Immunotherapy refers to any approach aimed at mobilizing or manipulating a patient's immune system to treat or cure disease[19], and immunological therapy by means of gene manipulation is based on the interaction between virus-based gene delivery systems and dendritic cells (DCs). Using viruses as vectors, it is possible to transduce DCs with genes encoding tumor-associated antigens (TAA), thus inducing a robust immune response[20,21].

A number of studies have established the role played by DCs in the immune system, and provided a rationale for using them as natural adjuvants for cancer immunotherapy[20-22]. Previous studies have concentrated on identifying the proliferating progenitors of DCs within the small CD34+ sub-fraction of cells in human blood[23]. These cells can be stimulated by cytokines (particularly by GM-CSF and TNF-alpha) to differentiate into DCs *in vitro* over a period of 1 wk[24]. It has also been more recently found that the combination of GM-CSF and IL-4 facilitates the generation of significantly larger numbers of DCs from monocytes/macrophages, which have equal or greater stimulatory activity in mixed lymphocyte reactions, and a greater capacity to present soluble protein antigens than CD34+ cell-derived DCs[23,24].

Gene manipulation

Gene manipulation transmits new genes/DNA into target cells infected with the viral vector, and has been most widely used to treat genetic diseases. The vector unloads its genetic material containing the therapeutic human gene into the target cell, which is finally restored to its normal state as a result of the generation of a functional protein encoded by the therapeutic gene[24,25]. The technique can be used in cancer to activate self and non-self antigens and enhance T cell responses. Some of the different types of viruses used as gene therapy vectors are listed in Table 2.

There are also various non-viral options for gene delivery. The simplest method is to introduce therapeutic DNA directly into target cells, but its application is limited by the fact that it can only be used with certain tissues and requires large amounts of DNA. Another non-viral approach involves creating a liposome (an artificial lipid sphere with an aqueous core), which is capable of shuttling the therapeutic DNA through the target cell's membrane, and a further delivery system is based on electroporation[25-28].

Problems in applying gene therapy

Whenever a foreign body (antigen, bacteria, *etc.*) enters the human tissue, the immune system is prompted to attack the invader, and so there is a risk of stimulating an immune response and reducing the effectiveness of gene manipulation. Furthermore, the immune system's enhanced response to previously encountered invaders makes it difficult for gene therapy to be repeated.

Viruses are the carriers of choice in most gene therapy studies, but they can give rise to a number of potential problems relating to toxicity, immune and inflammatory responses, gene control, and targeting. The main concern is that, once inside the patients, the viral vector may somehow recover its ability to cause disease, which is why we decided to use virus vectors with little or no replicative capacity, such as adeno-associated viruses (AAV)[29-31].

Viral delivery of antigen genes into dendritic cells

There are various ways of inserting antigen genes and proteins into DCs via protein pulses or viral vector loading[27-32]. Recombinant retroviruses, adenoviruses, and poxviruses can all efficiently transduce DCs[29-31],

Table 2 Commonest viruses used as gene therapy vectors

Retroviruses	Adenoviruses	Adeno-associated virus (AAV)	Herpes virus
8kb, RNA enveloped	35 kb, DNA, non-enveloped	5 kb, single stranded DNA, non-enveloped	61 kb, double-stranded DNA
Activate proto-oncogene by insertional mutagenesis	Episomal, transient	Stable integration, high infectivity	Infect mainly neurons
Cause lymphoma	Highly immunogenic, causing inflammation and anaphylactic shock	Non-pathogenic; requires helper viruses such as Adenoviruses for replication and packaging in mammalian cells.	Cause cold sores or blisters in the genital areas
Inactivation of transgene *in vivo*	One case of death	Long-term expression *in vivo*	Cutaneous skin lesions

but they all have well-known and serious disadvantages. Retroviruses can integrate chromosomally, but any residual contaminating wild-type virus can lead to significant disease and malignancy in the host. Furthermore, as they can also integrate gonadally and alter the germ line, their use may be restricted by the FDA[30,31].

Adenoviruses carry many genes in addition to the transgene, and the viral particle contains several proteins; the delivered antigen gene would therefore be only one of the many genes/proteins and epitopes to which a CTL response would be generated.

Unlike these viruses, AAVs are non-pathogenic, and various studies have shown that they are effective gene delivery vectors for both immortalized tissue culture cells and primary hematopoietic cells[33-36]. The helper-dependent parvovirus AAV can latently infect cells via stable chromosomal integration. Early studies demonstrated that 15-30% of immortalized cells could be latently infected with wild-type AAV, and the AAV genome was chromosomally integrated[21]. After the mapping of AAV genes and their functions[32-34], recombinant AAV virus vectors proved to have a similar capacity in immortalized tissue culture cells[33,34], and the recombinant AAV transduction of primary hematopoietic stem cells was achieved in 1988[35].

We have demonstrated that AAVs can be highly efficiently (>90%) used to transduce antigen genes into primary human monocytes (Mo) and Mo-derived DCs[36,37]. Unlike cells transduced using adenoviruses, retroviruses and other pathogenic viruses, AAV-transduced cells are not usually significant targets of the host immune system[37]. The use of the rAAV-based DC loading of human papillomavirus type 16, E6, and E7 antigen genes leads to robust and rapid antigen-specific, MHC class I-restricted CTL responses with one stimulation (one DC addition) and a 7-10 d co-incubation period[37,38]. Our data therefore strongly suggest that AAVs may be effective vectors for manipulating DCs[20,36,39].

DISCUSSION

Experimental algorithm for solid liver tumors

The key to the new evolution toward immunotherapy is to set up an algorithm for patients who will not respond to surgery. As shown in Figure 1, the liver tumor of selected patients is staged and the patients are directed to follow the clinical[8] or immunological algorithm. The experimental option is designed by taking specimens, with the tumor being preferably harvested laparoscopically or by means of the open technique. The next step is to insert modified antigens with carriers into the neoplastic cells. At this point, the choice is whether to inject them with DCs after leukopheresis, or by means of a virus (AAV). Both injections can be performed laparoscopically, thus allowing minimally invasive surgery, the introduction of the antigen inside the area of the tumor, and the initiation of a cell-mediated reaction designed to ensure immunological cytoreduction or debulking. An ultrasound-guided needle is placed into the abdomen, and a biopsy of the tumor can be taken.

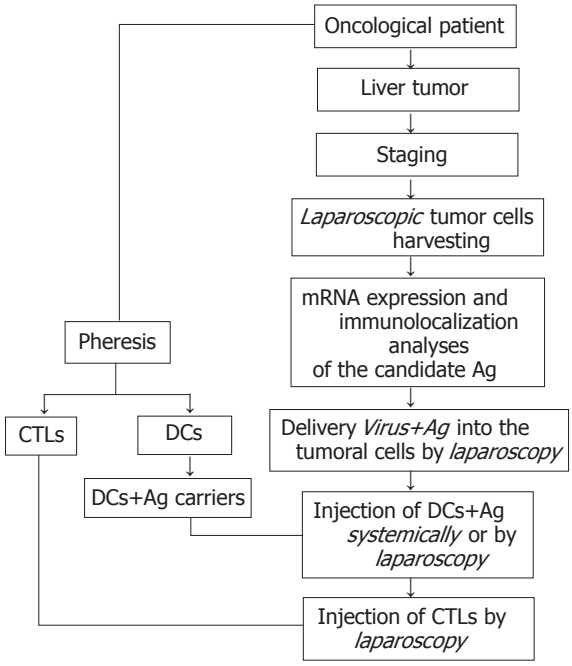

Figure 1 Relationships between laparoscopic surgery and immunotherapy for treatment of primary and secondary liver tumors. CTLs: cytotoxic T lymphocytes; DCs: dendritic cells; Ag: antigen.

As laparoscopy is a widely used surgical technique, most surgeons can easily adopt the procedure of injecting antigens, CTLs and DCs directly into the primary or secondary tumor in order to increase tumor immunogenicity and kill the remaining tumor cells by means of a local injection of CTLs and DCs for better cytoreduction. A port should be placed inside the internal jugular or subclavian artery in order to allow the retrieval of blood for leukopheresis; this port can be accessed quite easily using an external needle.

Improving cytoreduction: our new approach

The most widely used treatment is tumor resection, and so the main role of surgeons and oncologists is to decrease tumor bulk or mass in order to improve survival or allow the possibility of chemotherapy. Some liver tumors are so large that they are either inoperable or require such extensive surgery as to increase the incidence of death, but the introduction of cryoablation, alcohol injection, and radiofrequency ablation means that surgeons can reduce the amount of tumor in the liver, thus allowing chemotherapy to work on fewer tumor cells. The theory is that shrinking the tumor should lead to better chemotherapeutic results. Chemotherapy usually not only kills the tumoral cells remaining after radiofrequency, but also eliminates the satellite cells present in the liver or liver vessels together with good and normally replicating cells. We strongly believe that immunological therapy could become the new standard treatment for liver tumors. It not only debulks the tumor mass while destroying the tumor by means of a cell-mediated response, but its cell-

specific nature should enable it to kill satellite lesions and micro-metastases more efficiently (thus leading to better cytoreduction) without killing normal cells, and allow the use of low-dose chemotherapy to avoid or reduce undesirable side effects. Furthermore, the possible activation of memory responses could lead to much-needed long-term tumor immunosurveillance, which should reduce the incidence of relapses.

CONCLUSIONS

Evidence of practical immunotherapy treatment

Our initial clinical results suggest that there is an urgent need to explore further therapeutic options for liver tumors. This study introduces several innovations and a methodology that will help establish critical clinical assays for assessing immune responses targeting liver tissue, and verify the relationship between host response and liver tumor regression/progression.

Relevance to liver cancer research

We believe that immunological therapy can improve our overall understanding of how the host immune system interacts with primary and secondary liver cancer tissue, and will further elucidate useful methods for assessing this potential interaction.

Relevance to tumor immunology/immunotherapy

The importance of breaking host tolerance in order to achieve a tissue-specific mediated response and facilitate a favorable response to antitumor immunotherapy has recently been stressed in the literature[40]. For this reason, we believe that the use of a mini-invasive surgical approach, the immunotherapy and a clinical treatment will have a significant impact on liver tumors.

Costs and applications

Immunological therapy seems a very promising treatment for liver tumors. However, its specificity and cost makes it indicated for patients with tumors that cannot be resected by any of the different ablation methods, and small tumors in cirrhotic patients who are unsuitable candidates for standard surgery.

Improved cytoreduction

We believe that immunological therapy could become a new standard treatment of liver tumor for mainly three reasons: (1) it can debulk the tumor mass, while destroying the tumor by means of a cellular response; (2) it should be able to control the satellite lesions and micro-metastases more efficiently because of its more cell-specific nature, thus improving cytoreduction, avoiding the killing of normal cells, and reducing the use of chemotherapy and therefore its side effects; and (3) the possible activation of memory responses could lead to much-needed long-term tumor immunosurveillance, and thus reduce the incidence of relapses.

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• *REVIEW* •

Management of functional dyspepsia: Unsolved problems and new perspectives

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Abstract

The common characteristic criteria of all functional gastrointestinal (GI) disorders are the persistence and recurrence of variable gastrointestinal symptoms that cannot be explained by any structural or biochemical abnormalities. Functional dyspepsia (FD) represents one of the important GI disorders in Western countries because of its remarkably high prevalence in general population and its impact on quality of life. Due to its dependence on both subjective determinants and diverse country-specific circumstances, the definition and management strategies of FD are still variably stated. Clinical trials with several drug classes (e.g., proton pump inhibitors, H2-blockers, prokinetic drugs) have been performed frequently without validated disease-specific test instruments for the outcome measurements. Therefore, the interpretation of such trials remains difficult and controversial with respect to comparability and evaluation of drug efficacy, and definite conclusions can be drawn neither for diagnostic management nor for efficacious drug therapy so far. In view of these unsolved problems, guidelines both on the clinical management of FD and on the performance of clinical trials are needed. In recent years, increasing research work has been done in this area. Clinical trials conducted in adequately diagnosed patients that provided validated outcome measurements may result in better insights leading to more effective treatment strategies. Encouraging perspectives have been recently performed by methodologically well-designed treatment studies with herbal drug preparations. Herbal drugs, given their proven efficacy in clinical trials, offer a safe therapeutic alternative in the treatment of FD which is often favored by both patients and physicians. A fixed combination of peppermint oil and caraway oil in patients suffering from FD could be proven effective by well-designed clinical trials.

INTRODUCTION

Symptoms of upper gastrointestinal distress are of world-wide interest and very common in the general population. In developing countries the important form of dyspepsia is organic dyspepsia, whereas the problem of functional dyspepsia (FD) seems to be mainly confined to industrialized Western countries though convincing data for underdeveloped countries are still lacking[1]. It is estimated that the annual prevalence of recurrent upper abdominal discomfort in the United States and other Western countries is approximately 25%, about 2% to 5% of all primary care consultations are related to dyspeptic symptoms[2]. For many patients the symptoms are of short duration or mild severity[3] and are therefore self-manageable. Less than half of these patients consult their general practitioner[2]. Moreover, patients with upper gastrointestinal problems frequently suffer from recurrent affections. However, several long-term studies showed that high percentages of patients with dyspeptic symptoms at entry report similar symptoms of dyspepsia after some years[3,4]. Repetitive diagnostic measures and medical treatments with low success rates lead to high costs and frustrating results. Thus, FD represents not only a clinical challenge but also a major socio-economical problem. In recent years, a lot of efforts have been made by national and international consensus meetings to work out precise definitions as well as adequate management strategies for dyspepsia. Still unsolved problems and new perspectives for both research work and disease management in clinical practice are summarized and discussed in more detail in this review.

Definition of functional dyspepsia

Several definitions of dyspepsia have been proposed in the past decades[5] demonstrating the difficulties in categorizing

dyspepsia as a clearly pathologically defined entity based on the variability of symptoms. According to the proposition of an international committee meeting in Rome in 1991, the term "dyspepsia" refers to pain or discomfort centered in the upper abdomen[6] while discomfort refers to a subjective negative (or aversive) feeling that is distinct from pain. Discomfort may include several specific bothersome but non-painful symptoms, such as early satiety, fullness, bloating and nausea (the so-called Rome criteria). In Rome I and more recent Rome II reports[1,7-9], the symptoms of heartburn, acid regurgitation, and belching are excluded from the definition of dyspepsia because they are more likely related to gastroesophageal reflux disease (GERD) and aerophagia[1,9]. It is important to distinguish subjects with uninvestigated dyspepsia from patients with dyspepsia after adequate diagnostic procedure. Patients who have neither definite structural or biochemical explanation for their symptoms are considered to have FD. Thus, FD is defined as a persistent or recurrent dyspepsia for at least 12 wk in the preceding 12 mo if there is no evidence for organic disease (including upper endoscopy) that could cause the symptoms. The Rome II definitions of FD also exclude patients who report a relief of symptoms by defecation or symptoms associated with the onset of a change in stool frequency or stool form[9]. In the latter case, irritable bowel syndrome (IBS) is the diagnosis by definition. Coexistence of FD and IBS can be considered if there is pain or discomfort in the upper abdomen that is unrelated to bowel pattern and if there is other pain or discomfort that is related to bowel pattern[7].

Management of dyspepsia

Due to geographical, cultural, educational, social, and psychological aspects, universally applicable guidelines on diagnostic and therapeutical measures are difficult to implement[1,10]. Management strategies should be individualized and developed for each major community taking into account the prevalence of risk factors for gut diseases such as prevalence of *H pylori* infection, use of non-steroidal anti-inflammatory drugs, dietary habits, tobacco smoking and alcohol consumption[1,10]. Beyond these patient-related factors, the available financial and technical resources in each particular country may dictate the individual steps in the management of dyspepsia[1].

Nevertheless, useful recommendations regarding the management of dyspepsia are concluded in a recent systematic review of the literature[11]. To date, five management strategies can be offered to the physicians treating dyspeptic patients: (1) wait and see-strategy without diagnostic and therapeutic interventions; (2) empiric medical therapy with any subsequent investigation reserved for treatment failures; (3) immediate diagnostic evaluation in all cases; (4) testing for *H pylori* infection and reserving endoscopy for *H pylori*-positive cases to look for organic diseases (test-and-scope strategy); and (5) testing for *H pylori* infection by serology or urea breath test and treating all positive cases with *H pylori* eradication therapy (test-and-treat strategy).

For adult patients in Western countries with new onset of dyspepsia, endoscopy is the gold standard approach providing a firm diagnosis and facilitating decisions on treating or excluding organic diseases. In elderly patients or in those with alarm symptoms such as weight loss, immediate endoscopy is strongly advised. In respect of cost-effectiveness, a repeated endoscopy in those with an initially negative result should be avoided. An alternative management strategy in young dyspeptic patients under 45 years is non-invasive testing for *H pylori* infection and antibacterial treatment of positive cases[10-12]. Because of many substantial disadvantages such as antibiotic resistance, overtreatment, or undertreatment, there is ongoing discussion about the benefit of this strategy.

Management of functional dyspepsia

Patients with FD typically present an array of painful and non-painful symptoms demonstrating the multifactorial nature of this syndrome[13,14]. In order to identify pathophysiological abnormalities with subsequent targeted treatment and to promote more homogeneity, patients can be subdivided into ulcer-like, dysmotility-like and unspecified dyspepsia subgroups based on the concept of a cluster of symptoms[13,15]. Several studies have shown that this arbitrary classification seems to be unsustainable because of the considerable overlap of the subgroups, the lack of stability over time, and the inconsistent responses to therapy[13,16]. Currently, the existence of subgroups among dyspeptic patients is neither endorsed nor categorically disproved[7,8,13].

Another approach to a subdivision of patients with FD is the sus pected association with *H pylori* infection. Between 30% and 60% of patients suffering from FD have *H pylori*-induced gastritis. However, *H pylori* infection is also common in the asymptomatic background population[17,18]. Even most recent trials with prolonged follow-up, analyzing the association between *H pylori* status and specific symptom profiles in FD have produced inconsistent and conflicting results. To date, there is no convincing evidence for the relief of specific dyspeptic symptoms after an eradication therapy[5,13,19,20]. Thus, a benefit of anti- *H pylori* therapy in FD is not established [5,11,19].

Drug therapy for functional dyspepsia

The wide range of therapies reflects the uncertainty about the pathogenesis and the lack of satisfactory treatment. The pathophysiology of FD remains inadequately understood, even though various mechanisms may play a role in the development of symptoms. As yet, there is no cure for this disorder and available treatments are aimed at the relief of symptoms. Even though the efficacy of some currently established treatments (e.g., antisecretory agents or prokinetics) has been proven in placebo-controlled trials, these treatments yield sufficient relief of symptoms only in a proportion of patients[5].

In ulcer-like (pain predominating) functional dyspepsia, H_2-receptor antagonists have produced inconsistent response rates[21]. Patients with dysmotility-like symptoms

(upper abdominal discomfort predominating) may benefit from prokinetic drug treatment[22-24]. Proton pump inhibitors appear to be efficacious especially in patients with ulcer-like pain and accompanying reflux symptoms. The majority of controlled clinical trials have shown only minor advantages of these drugs compared to placebo[25,26].

Thus, efforts should be made to identify and develop new effective treatments. Various herbal medications are used in many countries for the treatment of patients with FD. While some clinicians believe that clinical experience appears to support the use of these remedies, randomized controlled studies supporting the efficacy of these treatments have been lacking in the past decades. Recently, several well-designed placebo-controlled clinical trials have provided evidence for the efficacy of herbal preparations used in the treatment of dyspepsia[27]. Particularly, patients with dysmotility-like dyspeptic symptoms, such as postprandial sensations of fullness, premature feelings of repleteness, non-acid eructation, or epigastric pain, experience a notable amelioration of their complaints[28,29].

Problems with evaluating drug efficacy in functional dyspepsia

Clinical trials in functional GI disorders remain a challenge due to a variable placebo response ranging 20-60%[30], marked spontaneous fluctuations of symptoms and a lack of widely accepted primary response variables. In addition, patients recruited at tertiary referral centers may represent a highly selected population that is less likely to respond to therapy[31]. It is likely that patients with FD present to general practitioners when their symptoms are worse. Therefore, spontaneous improvement may partially explain at least part of the placebo response[18].

Beside these well-known problems, the differences in the design of clinical drug trials in FD call for caution when interpreting their results. A systematic analysis of more than fifty eligible published placebo-controlled clinical trials testing prokinetics[32-35], cytoprotectives[36,37] or anti-ulcer agents[38-40] and other drugs[36,37] used in the treatment of functional dyspepsia revealed that single substantial items for the consistency of clinical studies such as inclusion and exclusion criteria for trial design and outcome measures are common but differ quite definitively in specific determinations[41]. Particularly, it is of importance how investigators deal with symptomatic GERD and other organic diseases. In 50% of the analyzed studies other upper GI disorders such as esophagitis and duodenal or gastric ulcer were not excluded; only 27% of the trials exclude or account for patients with overt irritable bowel syndrome as an overlapping functional disorder. The study design varies from parallel group, cross-over to multiple cross-over design[41]. The majority of analyzed trials fail to fulfill the indispensable requirement for efficacy evaluation and comparability of drug classes, i.e. use of clearly defined patient groups according to the consensual definition of FD and the use of validated outcome measures regarding described symptoms, their severity, and quality of life yielded with validated categorical and visual analog scales (VAS). Thus, the authors concluded that convincing conclusions for efficacious drug therapy in the treatment of FD cannot be drawn.

Promising outcome measures for clinical trials

Although some research work has been done to develop validated outcome measures of symptoms[42] which can be used in FD, no generally accepted scales are available. Categorical scales (often referred to as Likert Scales) and VAS (horizontal line, usually 10 cm with endpoints on which the patient must place a mark) have been extensively applied[29,39,43-45] and qualified as most eligible measurement scales by their reproducibility and ability to detect changes in a wide variety of clinical trials of different diseases. The usefulness of a reasonable combination of a categorical scale and a VAS is demonstrated by the dyspeptic discomfort score (DDS) which records the existence, frequency and severity of the symptoms of functional dyspepsia[28,29]. Integrating the dyspeptic, intestinal and extraintestinal autonomic discomforts assessed by means of numerical scales, the DDS seems to consider the entire complexity of this syndrome. Nevertheless, the DDS has not been validated yet.

A noteworthy measurement instrument to be mentioned is the clinical global impression (CGI) scale consisting of three items, namely severity of illness, global improvement and efficacy index. The first and second items are rated on a point scale while the third is a rating of the interaction of therapeutic effectiveness and adverse reactions. Originally conceived for schizophrenic studies, the CGI scale facilitates prognosis, survey and assessment of drug efficacy during the treatment period[28,29,44].

During the last years, attention has been drawn to the fact that in diseases without obvious biological or clinical markers such as functional dyspepsia, the use of quality of life instruments and psychometric documentation as an outcome measure can reflect treatment efficacy evaluated by its impact on symptoms as well as on patient well-being and functioning[41,46]. The underlying philosophy is that quality of life is affected by the severity of disease-specific symptoms. Hence, the reciprocal conclusion can be drawn by any change of symptom severity. Recently, validation data of the new disease-specific Nepean dyspepsia index (NDI)[46,47] and the quality of life in reflux and dyspepsia patient (QOLRAD) questionnaire[48] measuring frequency, intensity, and bothersomeness of upper gastrointestinal symptoms have been presented. The remarkable feature of the NDI is the consideration not only of a subject's ability to perform or engage in an aspect of life but also the enjoyment of that aspect of life. In a systematic review of full-length publications during 1980-2002 reporting studies in patients with FD and measuring health-related quality of life, none of the studies used dyspepsia-specific health-related quality of life instruments[49]. However, recently a first methodologically well-designed clinical study proving efficacy of the study drug by use of the NDI was reported by Holtmann and colleagues[50], which demonstrates a

statistically significant and clinically relevant superiority of a fixed combination of peppermint oil and caraway oil (PCC) in comparison to placebo. The reported outcome confirms the results formerly obtained with this herbal preparation in placebo-controlled clinical trials[28,44] and in a double-blind equivalence study with the prokinetic drug cisapride[29], measured by VAS, CGI and the DDS.

Recommendations for future trials

In view of the mentioned weaknesses in present trials, the most essential recommendations are summarized as follows.

According to the consensus for a diagnosis of FD, a minimum set of diagnostic measures including upper endoscopy, an abdominal ultrasound and basic laboratory is obligatory[6]. At the time of enrolment for a treatment study, eligible patients must have persistent symptoms that are of a sufficient degree to seek medical attention. Any definite structural abnormalities of the upper GI tract, explaining the symptoms, e.g., peptic ulcer confirmed by endoscopic evidence and biochemical agents such as daily use of NSAID or high dose aspirin must be excluded. To avoid an overlap with gastroesophageal reflux disease, patients in whom heartburn or acid regurgitation are the predominant symptoms or patients suffering from irritable bowel syndrome and other known organic diseases that might explain the dyspepsia symptoms must not be enrolled.

Despite some well recognized problems such as the occurrence of period-by-treatment interactions of cross-over trials resulting in ambiguous interpretation of data, the randomized, double-blind, placebo-controlled parallel group design is strongly advocated as the trial design of choice.

It is not to deny that even among physicians there is great variation in the definitions of common dyspeptic symptoms. In addition, terminology and possibly also the sensations experienced vary between cultures and countries. Therefore, it is advisable that clinical investigators use definitions of symptoms suggested by the Rome Working Party report and accommodated to common parlance in the respective study population.

As validated outcome measures like the NDI and the QOLRAD questionnaire are now available, their use is strongly recommended regarding described symptoms, their severity, and aspects of quality of life. In order to support the results obtained with these validated disease specific questionnaires, categorical scales, VAS and the CGI could be used as secondary outcome measures. Promising outcome measures such as DDS, should be validated soon in order to broaden the range of appropriate devices for evaluating drug efficacy in functional dyspepsia.

Further research using well-validated outcome instruments for measurement of individual symptoms as well as their severity and their impact on quality of life may perhaps result in a valid symptom-related categorization of functional dyspepsia that may be used to improve treatment strategies.

Causally determined by the aforementioned unsolved problems concerning the definition and management of FD as well as the listed weaknesses in trial methodology of present treatment studies, convincing conclusions for efficacious drug therapy cannot be drawn yet. However, it is very likely that effective drug therapies are av-ailable. Further research on well-validated measurement instruments for outcome data permitting comparability of drug classes may perhaps result in better insights with respect to effective treatment strategies. Quite recently, new perspectives have been arising from presented efficacy of a fixed peppermint oil/caraway oil preparation in a methodologically adequate clinical trial.

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Adenovirus expressing p27^{kip1} suppresses growth of established esophageal carcinoma xenografts

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Abstract

AIM: To investigate the growth suppression of adenovirus expressing p27^{kip1} on established esophageal tumors in nude mice.

METHODS: Esophageal carcinoma xenografts in nude mice were established by tumor tissue mass transplantation. The successfully constructed recombinant adenoviral vectors carrying p27^{kip1} gene (Ad-p27^{kip1}) were directly injected into the esophageal tumors in nude mice. Compared to control group, the growth curve of tumor was drawn and the growth inhibition rate of tumor was calculated. The histology of tumors was examined by hematoxylin and eosin (H&E) staining. The expression of p27^{kip1} and survivin was detected in tumors by immunohistochemical technique.

RESULTS: The growth of tumors in gene therapy group with Ad-p27^{kip1} was obviously suppressed compared to control group (0.42±0.08 g *vs* 1.17±0.30 g, t=6.39, P<0.01), the inhibition rate of tumor growth reached 64.1%. Pathological detection showed that the tumors in nude mice were poorly differentiated esophageal squamous carcinoma. In addition, the expression of p27^{kip1} was increased, while the expression of survivin was decreased in tumors after being transfected with Ad-p27^{kip1}.

CONCLUSION: p27^{kip1} gene therapy mediated by adenovirus vector has a significant inhibitory effect on esophageal carcinoma *in vivo*. Up-regulated p27^{kip1} expression and down-regulated survivin expression may be its important mechanisms.

Key words: p27^{kip1} gene; Esophageal carcinoma; Xenograft; Nude mice; Survivin gene

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INTRODUCTION

p27^{kip1} is an anti-oncogene with the function of negative regulation of cell cycle[1], and is also involved in the inhibitory reaction of cytokines, induction of cell differentiation and apoptosis, increase of cell adherence and regulation of resistance to drugs for noumenal tumors[2-6]. Our earlier investigation indicates that p27^{kip1} gene transfer mediated by adenovirus can obviously inhibit the growth of esophageal carcinoma cells[7]. Whether this gene therapy has the same effectiveness *in vivo* is worth further investigation. In this study, we explored the growth suppression of adenovirus expressing p27^{kip1} on established esophageal tumor in nude mice in order to find a new strategy for esophageal carcinoma therapy.

MATERIALS AND METHODS

Materials

The esophageal carcinoma cell strain EC109 and 4-week-old nude mice (Balb/C) of both sexes bred under specific pathogen-free conditions were purchased from Cancer Institute, Chinese Academy of Medical Sciences. pCMV5p27^{kip1} was presented by Dr. Gang Wang, Urinary Surgery Research Institute of the First Hospital of Beijing Medical University. pAACCMVpLpA and pJM17 were presented by academician Zu-Ze Wu, No. 2 Research Institute of Academy of Military Medical Sciences. DH$_{5a}$ was presented by Dr. Xu Peng, Heart Disease Department of the First Hospital of Beijing Medical University. Recombinant adenovirus was constructed by Molecular Biology Laboratory of Taihe Hospital. p27^{kip1}cDNA and adenovirus PCR primer were designed and synthesized by Saibaisheng Biological Company (Beijing, China). RPMI 1640 medium was purchased from Gibco BRL (NY, USA). Polyclonal goat antibody of survivin was purchased from Santa Cruz Biotechnology (CA, USA). Monoclonal mouse antibody of p27^{kip1}, ultra sensitive S-P kit, and 3,3-diaminobenzidine (DAB) kit were purchased from Fuzhou Maixin Biotechnology Co. Ltd (Fuzhou, China).

Construction of recombinant adenovirus Ad-p27^{kip1}

The process was the same as described in our previous work[7].

Cell culture

Human esophageal carcinoma cell strain EC109 was maintained in RPMI 1640 medium supplemented with 100 mL/L fetal calf serum (FCS), 100 kU/L penicillin, 100 mg/L streptomycin, 2 mmol/L L-glutamine, and 50 mL/L CO_2 in a humidified incubator at 37 ℃. The medium was changed every 2-3 d.

Establishment of esophageal carcinoma xenografts

EC109 cells growing exponentially were selected. The final concentration was adjusted to 10^7 cells/mL. Nude mice (Balb/C) of 4 wk old received injections into the dorsal midline in a 100 mL volume to establish tumors. The transplanted tumors were reproduced among the animals continually when the original grafts were growing well. Then esophageal carcinoma xenografts were established by transplanting the tumor tissue mass into the subcutaneous tissue of 36 nude mice. They were ready for use when the tumor diameter reached about 0.7 cm.

Therapeutic effect of intratumoral injection of Ad-p27^{kip1} into established tumors

The animals were randomized into three groups, and each group had seven mice with comparable tumor size within and among the groups. Intratumoral injection of Ad-p27^{kip1}, Ad-LacZ (1.0×10^{10} pfu) or PBS was made every other day for totally four times. The growth curve of tumor was drawn and the growth inhibitory rate of tumor was calculated after the animals were killed at wk 4. Tumor sizes were calculated by the formula: tumor volume = 1/2 ×length×width^2. The growth inhibitory rate of tumor was calculated by the formula: inhibitory rate = (tumor mass of control group–tumor mass of experimental group)/ tumor mass of control group.

Histology

The tumor tissues were fixed in 10% neutral formalin and embedded in paraffin. Sections of 5 μm thickness were used for morphological and immunohistochemical examinations. Paraffin sections were stained with hematoxylin and eosin (H&E) to demonstrate esophageal carcinoma tissue components.

Expression of p27^{kip1}

The paraffin sections were washed with phosphate-buffered saline (PBS, pH 7.4) and incubated in 3% hydrogen peroxide for 10 min to block endogenous peroxidase. After being heated for 10 min in 0.01 mol/L citrate buffer (pH 6.0) using a microwave oven, the sections were incubated with normal animal serum for 10 min and then with monoclonal mouse antibody of p27^{kip1} overnight at 4 ℃. Biotinylated antimouse immunoglobulin and streptavidin conjugated to horseradish peroxidase were subsequently applied. Finally, DAB was used for color development, and hematoxylin was used for counterstaining. As a negative control, the sections were processed in the absence of primary antibody. A scoring method was used to quantitate the p27^{kip1} expression in samples examined. A mean percentage of positive tumor cells was determined in at least five areas at 400-fold magnification. Samples with scores less than 50% were defined as low expression, otherwise as high expression[8]. These scorings were performed in a blinded fashion.

Expression of survivin

The sections carrying survivin protein were stained according to SP immunohistochemical staining method as aforementioned. The primary antibody was polyclonal goat antibody of survivin (dilution 1:200). The mean percentage of positive cells for the expression of survivin was determined in at least five areas at 400-fold magnification, and the samples with less than 10% positively stained cells were defined as negative. Samples with 10-29% positively stained cells were defined as +, 30-59% as ++, and 60% or more than 60% as +++[9].

Statistical analysis

The data were expressed as mean±SD. The difference between each group was analyzed by *t*-test. $P<0.05$ was considered statistically significant.

RESULTS

Growth suppression of established esophageal carcinoma xenografts by intratumoral injection of Ad-p27^{kip1}

Intratumoral injection of Ad-p27^{kip1} into established tumors induced partial growth suppression. The growth of tumors in gene therapy group with p27^{kip1} was obviously suppressed, being significantly different from that in control group and Ad-LacZ group ($P<0.01$). The growth inhibitory rate (IR) of tumor reached 64.1% (Figures 1 and 2, Table 1).

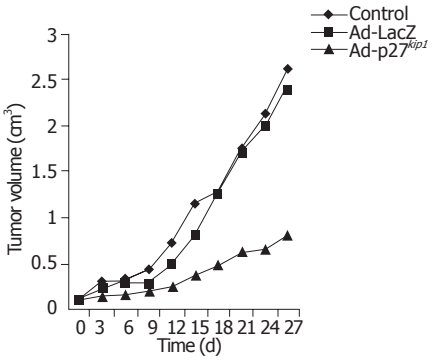

Figure 1 The growth curves of tumor.

Histological evaluation

The result of hematoxylin and eosin staining showed poorly differentiated esophageal squamous carcinoma (Figure 3).

Table 1 Tumor mass of three groups treated with Ad-p27^{kip1}, Ad-LacZ (1.0×10^{10} pfu) or PBS at wk 4

Groups (%)	Tumor mass (g)								IR
	1	2	3	4	5	6	7	mean±SD	
Control	1.02	0.93	1.31	1.47	0.86	1.63	0.95	1.17±0.30	
Ad-LacZ	0.72	1.36	1.12	0.87	0.92	1.23	0.85	1.00±0.23	
Ad-p27^{kip1}	0.32	0.46	0.35	0.43	0.57	0.38	0.40	0.42±0.08^{b}	64.1

$t = 6.39$, $^{b}P<0.01$ *vs* control group. IR: inhibition rate.

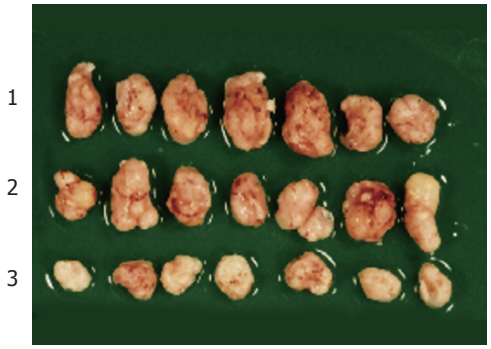

Figure 2 Growth suppression of Ad-p27^{kip1} on established esophageal carcinoma xenografts. 1: control group; 2: Ad-LacZ group; 3: Ad-p27^{kip1} group.

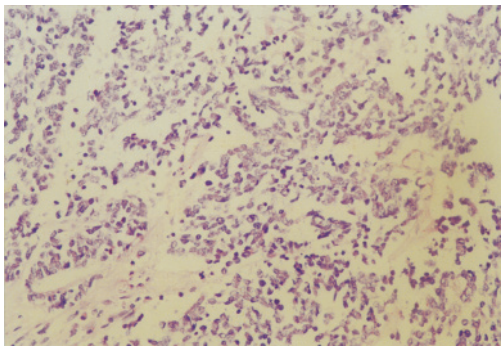

Figure 3 Hematoxylin and eosin (H&E) staining of established esophageal carcinoma xenografts (×200).

Expression of p27^{kip1}

Immunohistochemical staining showed that the expression of p27^{kip1} was increased in established esophageal carcinoma xenografts after being transfected with Ad-p27^{kip1} (Figure 4).

Expression of survivin

Survivin was prominently found in control group by immunohistochemistry and decreased in established esophageal carcinoma xenografts after being transfected with Ad-p27^{kip1} (Figure 5).

DISCUSSION

p27^{kip1} has been mapped to the short arm of chromosome 12 at the 12p12-12p13.1 boundary containing two introns and two exons[10]. Mutations of gene p27^{kip1} occur rarely in human tumors and the decrease in p27^{kip1} expression in tumor tissues is due to post-transcriptional degradation[11]. p27^{kip1} protein belongs to the family of proteins called cyclin-dependent kinase inhibitors (CDKIs). These proteins play an important role as negative regulators of cell cycle-dependent kinases during the progression of cell cycle. p27^{kip1} regulates the progression from G_1 into S phase by binding to and inhibiting the cyclin E/Cdk2 complex, which is required for entry into the S phase. It also interacts with various other cyclin complexes and is therefore designated as a universal CDKI[12-16]. p27^{kip1} expression decreases in esophageal cancer and may correlate with the histologic differentiation. Reduction of p27^{kip1} is considered to be an independent prognostic indicator of esophageal cancer[17-20].

In this study, we found that the growth of established esophageal carcinoma xenografts was obviously depressed and the inhibitory rate reached 64.1% after transfection with Ad-p27^{kip1}. The result of immunohistochemical staining demonstrated that Ad-p27^{kip1} could efficiently express p27^{kip1} in esophageal carcinoma. Ad-p27^{kip1} constructed in the present study is a kind of replication defective adenoviral vector, which has only one opportunity for infection in target cells without any dup-lication ability to fulfill the functions of adenoviral carrier, thus avoiding damage of adenovirus itself to target cells and reaching gene conversion.

We also observed that the expression of survivin was decreased in tumors of nude mice, indicating that p27^{kip1} might downregulate survivin expression. Survivin is a newly identified gene in inhibitors of apoptosis protein (IAP) family and is characterized by a unique structure with a single baculovirus IAP repeat and no zinc-binding domain known as Ring finger[21-23]. Survivin is an oncogene, which has been implicated in inhibition of apoptosis and control of mitotic progression. It is not usually detectable in normal adult tissues, but is prominently expressed in almost all common human cancers and most transformed cell lines. It is a short-lived protein degraded by ubiquitin proteasome pathway and interferes with the activation of caspases, called "cell death executioners"[24-28]. Disruption of survivin-microtubule interactions results in loss of survivin's anti-apoptosis function and increase of caspase-3 activity, a mechanism involved in cell death during mitosis. Survivin functions as a dimer and is regulated in a cell-cycle-dependent manner, peaking at G_2/M, nearly not detectable at G_1, and is associated with the mitotic spindle, centromeres, and the midbody in dividing cells[29-31]. p27^{kip1}, a down-regulating survivin may be associated with G1 blocking of p27^{kip1}, which has been identified in our previous studies[7].

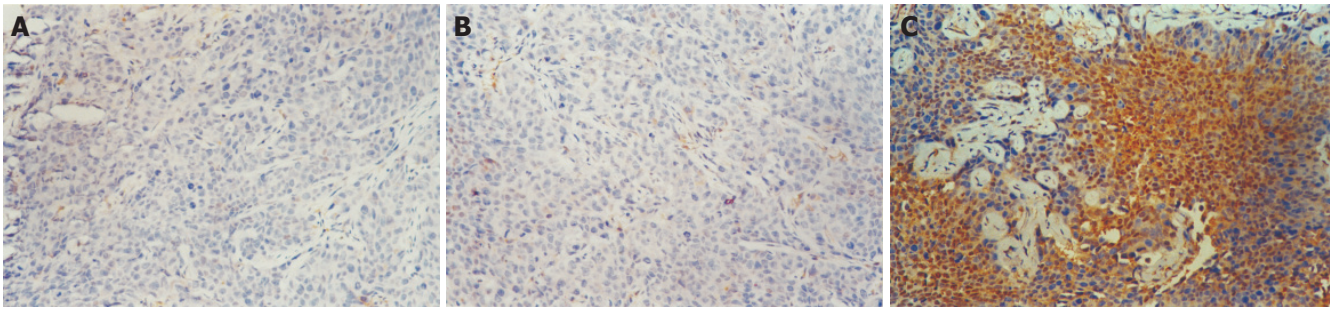

Figure 4 Result of immunohistochemical staining of tumors in nude mice. **A:** control group. p27^{kip1} protein located in cytoplasm of a few cells showing low expression; **B:** Ad-LacZ group. The result showed low expression also; **C:** Ad-p27^{kip1} group. p27^{kip1} protein located in cytoplasm and nucleus showing high expression (×200).

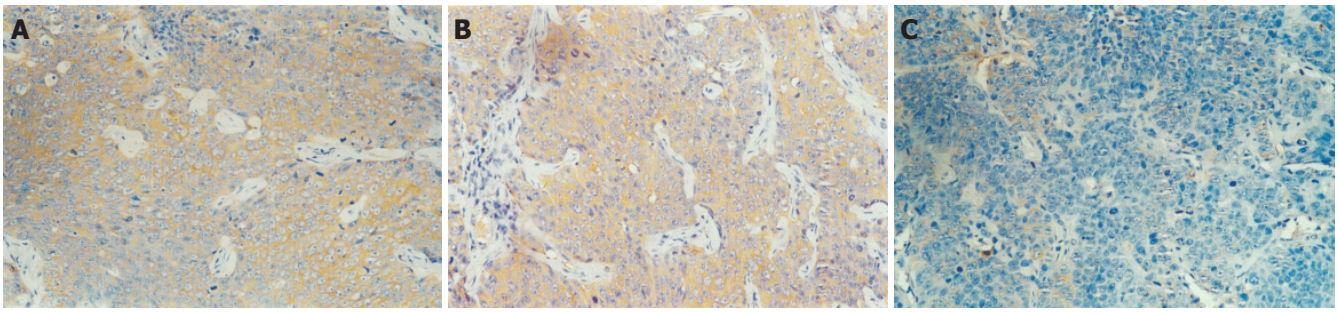

Figure 5 Result of immunohistochemical staining of tumors in nude mice. **A:** control group. Survivin protein located in cytoplasm showing high expression; **B:** Ad-LacZ group. The result showed high expression also; **C:** Ad-p27^{kip1} group. Survivin protein located in cytoplasm of a few cells showing low expression (×200).

In conclusion, p27^{kip1} gene therapy mediated by adenovirus vector has significant inhibitory effect on esophageal carcinoma *in vivo*. Upregulated p27^{kip1} expression and downregulated survivin expression may be its important mechanisms.

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Clinical significance of CT-defined minimal ascites in patients with gastric cancer

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Abstract

AIM: To study the clinical significance of minimal ascites, which was only defined by the CT and whose nature was not determined preoperatively, in the relationship with the peritoneal carcinomatosis.

METHODS: The medical records and the dynamic CT films of 118 patients with gastric cancer were reviewed. Factors associated with peritoneal carcinomatosis were analyzed in 40 patients who had CT-defined ascites of which the nature was surgically confirmed.

RESULTS: Only 12.5-25% of the CT-defined minimal ascites, whose volume was estimated to be less than 50 mL, were associated with peritoneal carcinomatosis. When the estimated CT-defined ascitic volume was 50 mL or more, peritoneal carcinomatosis was identified in 75–100%. When CT-defined lymph node enlargements were not found beyond the regional gastric area, perigastric invasions were not suspected, and the size of tumor was less than 3 cm, peritoneal carcinomatosis seemed significantly less accompanied at the univariate analysis. However, except for the minimal volume of CT-defined ascites in comparison with the mild or more, other factors were not confirmed multivariately.

CONCLUSION: In the patients with gastric cancer, CT-defined minimal ascites alone is rarely associated with peritoneal carcinomatosis, if it does not accompany other signs suggestive of malignant seeding. Therefore, consideration of active curative resection should not be hesitated, if CT-defined minimal ascites is the only delusive sign.

Key words: Ascites; Peritoneal carcinomatosis; Gastric cancer

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INTRODUCTION

Gastric cancer, quite uncommon in the developed countries, is still the second leading cause of cancer death in the world[1]. Surgical resection is the only effective therapy to secure curability of this fatal disease. Therefore, overestimation of the stage to render surgery being given up is critically hazardous, as it could deprive a patient of a chance for cure.

The two most frequent conditions in which gastric cancer is regarded incurable are when distant metastasis and malignant peritoneal seeding are demonstrated[2]. Dynamic CT is an excellent modality in clinical staging for gastric cancer and reliably detects metastasis to distant organs such as liver or lung[3,4]. However, the accuracy of dynamic CT in assessing malignant peritoneal involvement is somewhat questionable[4]. The presence of ascites, intestinal wall thickening, contrast-enhanced density in peritoneal adipose tissues, or implanted peritoneal, mesenteric or omental nodules are commonly stated CT findings suggestive of peritoneal carcinomatosis[5,6]. Among these, ascites is assumed to be the most frequently occurring clue for malignant seeding[5].

The nature of ascites is easily disclosed by aspiration cytology as long as the quantity of intra-abdominal fluid enables paracentesis[7,8]. Even small amount of ascites could be recovered for analysis by ultrasonography-guided needle aspiration[9,10]. However, dynamic CT has now become extremely sensitive and may occasionally detect subtle and equivocal amount of ascites in the pelvis, too little for preoperative aspiration-based examination. Without a cytological study or other strong evidences of peritoneal carcinomatosis, the significance of CT-defined minimal ascites might be ambiguous.

In clinical practice, some degree of hesitation is unavoidable in proceeding to surgery, when peritoneal seeding is equivocally suspected. The aim of this study is to make obvious whether the minimal ascites, which was

only defined by CT and whose nature was not practically feasible to characterize preoperatively, is related to genuine peritoneal carcinomatosis. We intend to draw a reasonable perception about 'minimal' ascites in view of clinical significance.

MATERIALS AND METHODS

Patients

Between January 2002 and December 2002, 118 consecutive patients were diagnosed for gastric cancer based on the histological examination of a gastroscopic biopsy and were also examined by dynamic CT at Boramae hospital. Their medical records and CT films were retrospectively reviewed. Out of these, 11 patients did not complete all necessary diagnostic and therapeutic procedures and were excluded from the study. One patient with massive ascites caused by decompensated hepatic cirrhosis was also excluded. Finally, a total of 106 patients remained for an initial analysis (BRM02) for overall frequency of ascites, peritoneal carcinomatosis, and distant metastasis.

The nature of ascites was often unexplored in the patients with metastatic diseases, because curative surgery was not indicated for them and thus characterization of ascites was practically unnecessary. Therefore, when analyzing the clinical implications of CT-defined ascites, we excluded 17 metastatic cases. In the remaining 89 cases (BRM02-NoMeta), the relationships between CT-defined ascites, surgery- or aspiration-recovered ascites, and peritoneal carcinomatosis were analyzed.

Thereafter, we tried to find factors predicting the absence of peritoneal carcinomatosis in the patients with CT-defined ascites, whose nature could not be evaluated preoperatively. To obtain sufficient number of cases for statistical analysis, we extended the study subjects to the cases of Boramae Hospital and of Seoul National University Hospital during the periods between March 1998 and December 2002. We reviewed 2 365 CT reports of the cases who had undergone open abdominal surgery, so whose ascitic natures were confirmed. In this screening step based on the reports, we collected 40 operated cases in which the patients had CT-defined ascites of undetermined nature at preoperative phase and had no definite evidence of distant metastasis or apparent peritoneal seeding (BRM-SNU group). Their medical records and CT films were reviewed in detail.

Radiologic analysis

Dynamic CT studies were performed with Somatom plus-4 scanner (Siemens Medical System, Erlangen, Germany). The abdomen and pelvis were scanned with helical technique and the image was reconstructed at 1-cm-thick sections. Patients were asked not to drink or eat anything for 8 h before CT examination. A total of 500-1 000 mL of tap water was given by mouth, immediately before scanning. A total of 80-120 mL of iopromide contrast medium (Ultravist370®, Schering, Berlin, Germany) was administrated by Mark V dedicated CT injector (Medrad, Pittsburgh, PA, USA) at a flow rate of 3 mL/s, through 18-gauge angiographic catheter placed in the antecubital vein.

Ascites was defined at CT images by at least two experienced radiologists, when the reasonably low radiologic density of 10 or less Hounsfield number was found within the pelvic cavity outside intra-abdominal or pelvic organs. Volume of ascites was estimated by the ruler grids applied on CT images. For example, the area of ascites was measured about 3.5 cm^2 in Figure 1A and 16 cm^2 in Figure 1B and these respectively corresponded with the estimated volumes of 3.5 mL (3.5 $cm^2 \times 1$ cm) and 16 mL (16 $cm^2 \times 1$ cm), because the interval between the serial images obtained in the study was 1 cm. When fluid densities were detected in more than one image, the volumes at each image were added up.

'Minimal' ascites was defined, when the volume of ascites was estimated to be less than 50 mL, and ascites of 50-300 mL was defined as 'mild'. Because we focused on the small amount of ascites which could not be easily evaluated by conventional measures at the preoperative stage, definition about moderate to severe ascites was not considered.

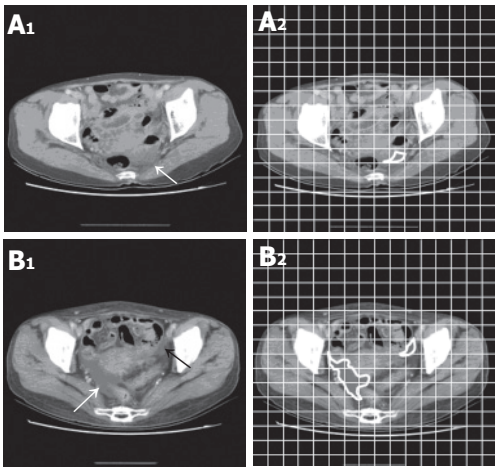

Figure 1 To estimate the volume of ascites by applying grids. Arrows indicate ascitic density (**A1-2**-**B1-2**).

Obliteration of a fat plane between the stomach and adjacent organs or apparent infiltration shown in the CT images was regarded as tumor invasion. Lymph nodes were considered significantly enlarged, when the long diameter was more than 1 cm. The lymph nodes were classified as 'regional' when they were located along the lesser or the greater curvatures of stomach, or at the left gastric, common hepatic, celiac, or splenic arteries. Other intra-abdominal nodes beyond these regions, such as the hepatoduodenal, retropancreatic, mesenteric, or para-aortic area, were defined as distant lymph nodes.

Statistical analysis

Data were analyzed by SPSS (11.5th version) software. Chi-square test was used for categorical data analysis, and the

univariate analysis and the multivariate logistic regression modeling were employed for assessing the predictive factors for the absence of peritoneal carcinomatosis in patients with CT-defined ascites.

RESULTS

Overall proportion of CT-defined ascites, peritoneal carcinomatosis and distant metastasis in the patients with gastric cancer (BRM02 group)

One hundred and six patients committed to the initial analysis consisted of 70 men and 36 women with a mean age of 63.3 years. The stage of gastric cancer at diagnosis was stage I, 23.6%; stage II, 7.5%; stage III, 34.0%; and stage IV, 34.9%. Patients with early gastric cancers were 27.4%.

Of the BRM02 patients, 22 (20.7%) had ascites defined by CT images. Based on the estimated volume of ascites, 12 patients belonged to the category of minimal ascites and 10 patients had mild or more ascites (11.3% and 9.4% of all patients, respectively).

Twenty patients out of the 106 BRM02 group (18.9%) were found to have peritoneal carcinomatosis that was confirmed by preoperative aspiration cytology or laparotomy no matter, whether they had CT-defined ascites or not. Seventeen patients (16%) had metastatic diseases to distant organs. Among these, four patients (3.8%) were confirmed to have both distant metastasis and malignant peritoneal seeding (Table 1).

Clinical realities of CT-defined ascites in the metastasis-free patients (BRM02-NoMeta)

Out of the remaining 89 patients (BRM02-NoMeta) after excluding those with metastatic diseases, 8 had minimal ascites, and 7 had mild ascites. There were no patients having ascites greater than mild category in the metastasis-free group. The nature of ascites was explored by surgery, in all 8 cases with minimal ascites, and in 5 out of 7 cases with mild ascites. The remaining two patients' ascites were proven malignant by aspiration cytology, and surgery was not performed.

Ascites defined by CT images were not always identified as peritoneal fluid on surgery. As for the CT-defined minimal ascites, ascitic fluid was recovered at the surgical field in only one (12.5%) of the cases. However, as CT-estimated ascitic volume was higher, surgical correlation improved. In the cases of 'mild' ascites which had more volume than 'minimal', the concordance rate between CT-defined ascites and surgery- or aspiration-recovered ascites was as high as 85.7% (6 out of 7). On the other hand, 5 (6.8%) out of 74 patients who had no CT-identified ascites preoperatively demonstrated ascites at the time of surgery (Table 2A).

The relationship between CT-defined ascites and peritoneal carcinomatosis was analyzed (Table 2B). Among the patients who did not show ascites at the CT images, seven (9.5%) had peritoneal carcinomatosis on surgery. Seven out of sixteen (43.8%) patients with peritoneal carcinomatosis did not demonstrate radiological signs of ascites at the preoperative CT. In the patients with CT-defined 'minimal' ascites, only 25% were concluded by surgery to have peritoneal carcinomatosis. This was contrast to the cases with CT-defined 'mild' ascites, in which all of the CT-defined ascites turned out to have occurred in the association with peritoneal carcinomatosis.

The presence of surgery- or aspiration-recovered ascites was not always accompanied with malignant peritoneal seeding, too. Four out of twelve patients (33.3%) with surgically or cytologically recovered ascites did not accompany peritoneal carcinomatosis, and 8% of ascites-free patients were actually positive for malignant peritoneal seeding (Table 2C).

Factors favoring absence of peritoneal carcinomatosis

Table 1 Proportion of peritoneal carcinomatosis, distant metastasis, and CT-defined ascites in the patients with gastric cancer that completed diagnostic work-up including dynamic CT in January 2002–December 2002 at Boramae Hospital (BRM02)

	Peritoneal carcinomatosis			
Distant metastasis	Negative	Positive	Unknown	Total
Negative	73 (67/6/0)[1]	11 (6/2/3)	0	84 (73/8/3)
Positive	5 (4/1/0)	4 (1/0/3)	8 (5/3/0)	17 (10/4/3)
Unknown	0	5 (1/0/4)	0	5 (1/0/4)
Total	78 (71/7/0)	20 (8/2/10)	8 (5/3/0)	106 (84/12/10)

[1]CT-defined ascites (none/minimal/more than minimal).

Table 2 Clinical realities of CT-defined, preoperatively malignancy-undetermined ascites in the metastasis-free patients (BRM02-NoMeta)[1]

a

	Surgery- or aspiration-recovered ascites			
CT-defined ascites	Negative (%)	Positive (%)	Unknown (%)	Total (%)
No	68 (91.9)	5 (6.8)	1 (1.4)	74 (100)
Minimal	7 (87.5)	1 (12.5)	0 (0.0)	8 (100)
Mild	0 (0.0)	6 (85.7)	1 (14.3)	7 (100)
Total	75	12	2	89

b

	Peritoneal carcinomatosis		
CT-defined ascites	Negative (%)	Positive (%)	Total (%)
No	67 (90.5)	7 (9.5)	74 (100)
Minimal	6 (75.0)	2 (25.0)	8 (100)
Mild	0 (0.0)	7 (100)	7 (100)
Total	73	16	89

c

	Peritoneal carcinomatosis		
Surgery- or aspiration-Recovered ascites	Negative (%)	Positive (%)	Total (%)
Negative	69 (92.0)	6 (8.0)	75 (100)
Positive	4 (33.3)	8 (66.7)	12 (100)
Unknown	0 (0.0)	21 (100)	2 (100)
Total	73	16	89

[1]Presence of ascites was not examined because operation was immediately ceased following observation of malignant omental cakes.

in the patients with CT-defined ascites which was not yet determined whether malignant or not.

We tried to search factors favoring absence of peritoneal carcinomatosis in the gastric cancer patients with CT-defined ascites in the larger number of cases. The sex and age distributions in these 40 patients (BRM-SNU) were not statistically different from those of the initial 106 gastric cancer patients (BRM02).

Majority of CT-defined ascites, of which the nature could not be preoperatively characterized, had volumes of less than 10 mL with a left-skewed pattern (Skewness = 2.768, Figure 2). The range of volume was 1-300 mL; the mean was 34.8 mL and the median was 9.9 mL. Thirty-two cases were categorized as 'minimal' ascites and the rest eight were 'mild' ascites. Age and sex were not related to the volume of CT-defined ascites (data not shown), but the stage of cancer seemed lower in the patients with minimal ascites than those with mild ascites (Table 3).

The CT-defined 'minimal' ascites were associated with peritoneal carcinomatosis in only 12.5% and demonstrated surgically recovered ascites in only 9.4% (Table 4). These data confirmatively reproduced the results obtained from the fewer cases in BRM02 (Table 2). Contrary to the CT-defined 'minimal' ascites, the CT-defined 'mild' ascites were accompanied with peritoneal carcinomatosis in as high as 75%, and were proved to have genuine fluid collection in 62.5%.

In addition to the ascitic volume, several other factors suggestive of negative peritoneal carcinomatosis were tested by the univariate analysis in the patients with CT-defined minimal or mild ascites (Table 5). When enlargement of peritoneal lymph nodes were absent or confined to the regional perigastric area at the CT images, the negative predictive value for peritoneal carcinomatosis was 88.9%. Free of CT-defined perigastric invasion also negatively predicted peritoneal carcinomatosis in 90.9%. None of the patients with tumors with less than 3 cm were accompanied with malignant peritoneal seeding. However, except for the CT-defined 'minimal' ascites in comparison with the more abundant ascites, none of the above factors obtained statistical significance by multivariate analysis as an independent predictor for absence of malignant seeding.

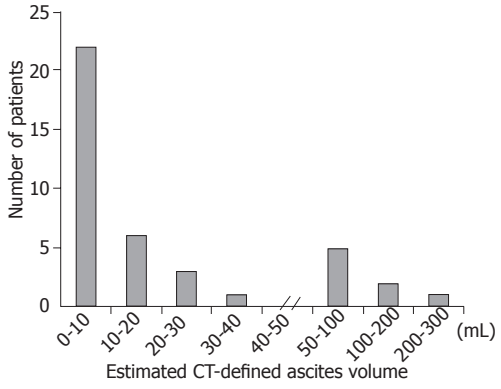

Figure 2 Frequency of patients based on the estimated CT-defined ascites volume.

Table 3 Proportion of the minimal or mild CT-defined ascites at each UICC stage of gastric cancer

Stage	Minimal ascites (%)	Mild ascites (%)
1	9 (28.1)	0 (0)
2	4 (12.5)	1 (12.5)
3	5 (15.6)	1 (12.5)
4	14 (43.8)	6 (75.0)
Total	32 (100)	8 (100)

DISCUSSION

CT has been established as the most popular staging modality in gastric cancer although conventional or endoscopic ultrasonography, or laparoscopy can also be used[3,4,10-14]. The role of CT in detecting distant metastasis has been particularly well recognized; overall sensitivity for assessing liver metastasis reached about 62-89%[4,13,14].

However, CT is less reliable in identifying ascites or peritoneal carcinomatosis; the sensitivity was merely

Table 4 Peritoneal carcinomatosis and surgically recovered ascites in the patients with minimal or mild CT-defined ascites

CT-defined ascites	Peritoneal carcinomatosis		Significance	Predictive value
	Negative	Positive		
Minimal	28 (27/1)[1]	4 (2/2)		NPV: 87.5%[2]
Mild	2 (1/1)	6 (2/4)	$P<0.01$	

[1]Surgically recovered ascites (negative/positive). [2]NPV: negative predictive value of CT-defined 'minimal' ascites for peritoneal carcinomatosis, in comparison with CT-defined 'mild' ascites.

Table 5 Peritoneal carcinomatosis in the patients with CT-defined, yet preoperatively malignancy-undetermined ascites. Univariate analysis

	Peritoneal carcinomatosis		Significance	Predictive value
	Negative	Positive		
CT-defined enlarged lymph node (L/N)				
None or Regional L/N	24	3		NPV: 88.9%[1]
Distant L/N	6	7	$P<0.01$	
CT-defined perigastric invasion				
Negative	20	2		NPV: 90.9%
Positive	10	8	$P<0.05$	
Tumor size defined by endoscopy or UGIS				
<3 cm	10	0		NPV: 100%
3-10 cm	16	6		
>10 cm	4	4	$P<0.05$	
EGC *vs* AGC[2]				
EGC	7	0		NPV: 100%
AGC	23	10	NS	

[1]NPV: negative predictive value for peritoneal carcinomatosis. [2]EGC: early gastric cancer; AGC: advanced gastric cancer.

36-46.7% for ascites and was 13-30% for malignant peritoneal seeding[4,12,14,15]. While the full blown peritoneal carcinomatosis demonstrating all the relevant radiologic features is diagnosed unambiguously, early and tiny malignant implantation cannot be easily decided. Although ascites has been regarded an important sign suggesting peritoneal carcinomatosis[5], the meaning of ascites may become ambiguous, as the dynamic CT detects subtle amounts of peritoneal fluid collection with increased sensitivity.

Then, what is the clinical significance of minimal ascites found in the CT image? Our data showed that minimal amounts of suspicious ascites defined by the dynamic CT were associated by peritoneal carcinomatosis in only 12.5-25%. This data may suggest that the patients need not hesitate to go through surgery, if the CT-defined minimal ascites is the only delusive clue for peritoneal seeding.

In the past, these amounts of minimal ascites might have never been detected by the CT and, therefore, might have never become a clinical issue. It may be argued whether the CT-defined minimal ascites is true or false-positive. However, this kind of question seems clinically out of point. Confirmation of real ascites may hardly be possible. Even surgery cannot be a gold standard in judging the presence of ascites because some blood or irrigated saline might be inevitably mixed with minimal, if any, ascites during surgery. Moreover, peritoneal cavity physiologically contains small amount of serous fluid which has been produced by permeable mesothelium[16], although this disperses diffusely in the peritoneal cavity and is not usually detected by the CT. It remains uncertain, if our CT-defined 'minimal' ascites is exaggerated physiologic fluid, or pathologic ascites.

On the other hand, high index of suspicion may have rendered observers to detect phantom radiologic ascites in the cancer patients. However, an article by Chen *et al*. also reported that small amounts of ascites were detected at the perigastric area in 39% of patients with gastric cancers by endoscopic ultrasonography and they were not significantly correlated with macroscopic peritoneal carcinomatosis[17]. Revealing the mechanism in the development of minimal ascites may be beyond the scope of this study.

No matter whether CT-defined minimal ascites is true or false, the apparent existence of minimal ascites reasonably defined by the dynamic CT may confuse a physician in determining the patient's operability. This may be particularly critical in the patients with marginally poor condition. It is possible that, for example, elderly patients may give up radical surgery, when they are imprudently suggested the worse prognosis, because of the suspicious ascites. Adoption of laparoscopic staging in patients with CT-defined minimal ascites is another issue. Although laparoscopic examination may reveal the nature of minimal ascites more clearly[12], this procedure is not always routinely available at every hospital. In the most common practice setting, a physician cannot help but judge the operability according to the CT finding and other clinical manifestations.

If CT-defined minimal ascites has just low probability of peritoneal carcinomatosis, what additional factors could enhance the possibility of free peritoneum? Of course, it should be explored first whether peritoneal nodules, fat strands, pleated soft tissue, thickening of omental, mesentery and/or bowel wall, or other radiologic clues for malignant seeding were accompanied with or not. As our data suggested, when CT-defined lymph node enlargements were not found beyond the regional gastric area, CT-defined perigastric invasions were not detected, and the size of tumor was less than 3 cm, the probability of true peritoneal carcinomatosis may be very low, at least based on the univariate analysis.

In conclusion, majority of malignancy-undetermined CT-defined minimal ascites that was estimated to be less than 50 mL at the preoperative phase are not significantly related to the peritoneal carcinomatosis. Therefore, if the peritoneal carcinomatosis was not definitely established, passive therapeutic strategy should not be applied to those patients simply because ascites is suspected. Definite meaning of CT-defined minimal ascites may need to be reinterpreted by the final effect on survival after long-term follow-up, and this study is under way.

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• *GASTRIC CANCER* •

Polymorphisms of DNA repair genes *XRCC1* and *XRCC3*, interaction with environmental exposure and risk of chronic gastritis and gastric cancer

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Abstract

AIM: To evaluate the association between polymorphisms *XRCC1* Arg194Trp and Arg399Gln and *XRCC3* Thr241Met and the risk for chronic gastritis and gastric cancer, in a Southeastern Brazilian population.

METHODS: Genotyping by PCR-RFLP was carried out on 202 patients with chronic gastritis (CG) and 160 patients with gastric cancer (GC), matched to 202 (C1) and 150 (C2) controls, respectively.

RESULTS: No differences were observed among the studied groups with regard to the genotype distribution of *XRCC1* codons 194 and 399 and of *XRCC3* codon 241. However, the combined analyses of the three variant alleles (194Trp, 399Gln and 241Met) showed an increased risk for chronic gastritis when compared to the GC group. Moreover, an interaction between the polymorphic alleles and demographic and environmental factors was observed in the CG and GC groups. *XRCC1* 194Trp was associated with smoking in the CG group, while the variant alleles *XRCC1* 399Gln and *XRCC3* 241Met were related with gender, smoking, drinking and *H pylori* infection in the CG and GC groups.

CONCLUSION: Our results showed no evidence of a rela-tionship between the polymorphisms *XRCC1* Arg194Trp and Arg399Gln and *XRCC3* Thr241Met and the risk of chronic gastritis and gastric cancer in the Brazilian population, but the combined effect of these variants may interact to increase the risk for chronic gastritis, considered a premalignant lesion. Our data also indicate a gene-environment interaction in the susceptibility to chronic gastritis and gastric cancer.

Key words: Gastric cancer; Gastritis; *XRCC1*; *XRCC3*; Polymorphism; Environmental exposure

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INTRODUCTION

DNA repair pathways are responsible for maintaining the integrity of the genome in face of environmental insults and general DNA replication errors, playing a role in protecting it against mutations that lead to cancer[1]. So, polymorphisms of DNA repair enzymes, which may alter the function or efficiency of the DNA repair, may contribute to an increased risk of environmental carcinogenesis[2]. These low-penetrance susceptibility genes have common variants and interact with environmental factors, contributing as a major factor to the populational incidence of cancer[3]. Several polymorphisms in genes that participate in different DNA repair pathways, such as *XPD*, *XPF*, *ERCC1*, *XRCC1*, *XRCC3*[4], *hOGG1*[5], *XPA*, *XPB*[6] and *XPC*[7], have been identified and related to cancer susceptibility.

The *XRCC1* gene is responsible for a scaffolding protein that directly associates with other proteins such as DNA polymerase β, PARP (ADP-ribose polymerase) and DNA ligase III in a complex, to facilitate the processes of base excision repair (BER) or single-strand break repair[8]. The BER pathway repairs DNA damage caused by a variety of endogenous and exogenous factors, including oxidation, alkylating agents and ionizing radiation[1,9]. The *XRCC1* protein can bind directly to both gapped and nicked DNA, as well as to gapped DNA associated with DNA

polymerase β, suggesting that this protein might be independently involved in DNA damage recognition[10]. Two polymorphisms, more often found in *XRCC1'* conserved sites, lead to a C→T substitution at codon 194 in exon 6 and to a G→A substitution at codon 399 in exon 10 of the gene, leading to the amino acid alterations arginine (Arg) to tryptophan (Trp) and arginine (Arg) to glutamine (Gln), respectively. These changes in conserved protein sites may alter the BER capacity, increasing the chances of DNA damage[4].

The Arg399Gln variant is more frequent and has been associated mainly with head and neck[11], colorectal[12], gastric[13], esophageal[14,15], breast[16] and lung[17,18] cancers. The Arg194Trp polymorphism has been related to colorectal[12], gastric[13], head and neck[19] and skin[20] cancers.

Protein *XRCC3* functions in the DNA double-strand break (DSB) and cross-link repair[21] and interacts and stabilizes Rad51[22], one of the key components of the homologous repair (HR) pathway. The HR pathway uses a second intact copy of a homologous chromosome as a template to copy the information lost at the DSB site, resulting in a high-fidelity process and preventing chromosomal aberrations[9]. The main polymorphism in this gene involves the change of threonine (Thr) to methionine (Met) at codon 241 in exon 7[4]. Little is known about the functional consequences of this variation, although some studies observed a positive relation between the Thr241Met polymorphism and an increased risk for skin[23], bladder[24], breast[25] and lung[26] cancers.

So far, the investigations about interactions between *XRCC1* and *XRCC3* polymorphisms and environmental carcinogenesis have produced scarce and conflicting results[27,28,29,30], showing the functional complexity of these variants, that can include their interaction with environmental factors, thus modulating the susceptibility to cancer. Regarding gastric cancer, only a few studies were conducted to investigate its association with *XRCC1* and *XRCC3* variants[13, 27,29,31].

In Brazil, gastric cancer is still one of the most frequent types of cancer. The estimate for 2005 points to the fourth place in incidence and mortality, with about 23 000 new cases and 12 000 deaths[32]. However, multiple factors are thought to play a role in gastric carcinogenesis, including diet[33], lifestyle[34], pathological changes in the stomach such as chronic gastritis[35], and genetic alterations[36,37], besides the infection by *Helicobacter pylori*, the first bacterium to be termed as a definitive cause of cancer[38].

Thus, we conducted a study to evaluate the association between the polymorphisms *XRCC1* Arg194Trp and Arg399Gln and *XRCC3* Thr241Met and the risk of chronic gastritis and gastric cancer in a Brazilian population, as well as the interaction between these polymorphisms and environmental factors involved in gastric carcinogenesis.

MATERIALS AND METHODS

Subjects

This was a case-control study on chronic gastritis and gastric cancer. The case groups comprised 202 patients with a histopathologically confirmed diagnosis of chronic gastritis (100 men and 102 women), with a mean age of 52 years (range 19-86 years), and 160 patients with a histopathologically confirmed diagnosis of gastric adenocarcinoma (118 men and 42 women), with a mean age of 61 years (range 28-93 years). All subjects were recruited from the Hospital de Base in São José do Rio Preto, SP, and from the Pio XII Foundation in Barretos, SP, Brazil. Gastric adenocarcinomas were classified as diffuse or intestinal types, according to the classification proposed by Lauren[39], and the chronic gastritis cases according to the Sidney System[40]. *H pylori* infection was histologically established by the Giemsa staining technique. Two cancer-free control groups with no previous history of gastric disease were matched to the case groups with respect to age, gender and ethnicity. The control group for chronic gastritis (C1) was composed of 202 healthy individuals (100 men and 102 women) with a mean age of 51 years (range 20-85 years), and the control group for gastric cancer (C2) consisted of 150 healthy volunteers (108 men and 42 women) with a mean age of 59 years (range 22-93 years). Epidemiological data on the study population were collected using a standard interviewer-administered questionnaire, with questions about current and past occupation, smoking habits, alcohol intake and family history of cancer. This work was approved by the National Research Ethics Committee and written informed consent was obtained from all individuals.

DNA extraction and genotype analyses

About 5 mL of whole blood were collected from all study participants in sterile EDTA-coated vacutainers. The samples were assigned a unique identifier code. DNA was extracted according to Abdel-Rahman *et al.*[41] and stored at -20 ºC until used for genotyping.

Genotypic analyses of the *XRCC1* gene were carried out by multiplex PCR-RFLP, using primers for codons 399 (F 5' -TTGTGCTTTCTCTGTGTCCA-3' and R 5'-TCCTCCAGCC TTTTCTGATA-3') and 194 (F 5'-GCCCCGTCCCAGGTA-3' and R 5'-AGCCCCAAGACCCTTTCACT-3'), which generate a fragment of 615 and 491 bp, respectively, as previously described[12] with modifications. Briefly, PCR was performed in 25 μL reaction buffer containing 12.5 pmol each primer, 0.2 mmol/L of dNTPs, 3 mmol/L of $MgCl_2$, about 100 ng DNA and 1 U of Taq DNA polymerase. The PCR products were digested overnight with 10 U of *Msp*I at 37 ºC. The wild-type Arg allele for codon 194 is identified by the presence of a 293 bp band, and the mutant Trp allele by the presence of a 313 bp band (indicative of the absence of the *Msp*I cutting site). For codon 399, the presence of two bands of 375 and 240 bp, respectively, identifies the wild-type Arg allele, while the uncut 615 bp band identifies the mutant Gln allele (indicative of the absence of the *Msp*I cutting site). A 178 bp band, resulting from an additional invariant *Msp*I cutting site in the 491 bp amplified fragment, is always present and serves as an internal control for complete enzyme digestion.

Polymorphism of the *XRCC3* gene was deter-

mined by PCR-RFLP, using codon 241 primers (F- 5' GCCTGGTGGTCATCGACTC 3' e R- 5' ACAGGGCTCTGGAAGGCACT GCTCAGCTCACGCACC 3'), as previously described by David-Beabes *et al.*[42]. The 25 µL PCR mixture contained about 100 ng of DNA, 12.5 pmol of each primer, 0.2 mmol/L of dNTPs, 2 mmol/L of $MgCl_2$ and 1 U of Taq DNA polymerase. The 552 bp amplified product was digested overnight with 5 U of *Nla*III at 37 ºC. The wild-type allele Thr was identified by the presence of two 239 and 313 bp bands, while the mutant allele Met was represented by 105, 208, and 239 bp bands.

Statistical analysis

Chi-square or Fisher's exact tests were utilized to compare the groups with regard to genotype frequencies and putative risk factors such as age, gender, ethnicity, smoking, drinking, *H pylori* infection and histological type of adenocarcinoma. To investigate the gene-environment interactions, the odds ratios (OR) and their 95% confidence intervals (95% CI) were calculated, according to a combination of the *XRCC1* and *XRCC3* polymorphisms with putative risk factors. The statistical analyses were performed using Statidisk and GraphPad InStat computer software programs. A probability level (*P*) of less than 0.05 was used as criterion of significance.

RESULTS

Cases and controls did not show any statistically significant difference with regard to age, gender and ethnicity, indicating a well-matched study population. Gastric cancer (GC) patients were more likely to be cigarette smokers or alcohol drinkers than chronic gastritis (CG) patients and controls (C2), and this difference was statistically significant ($P<0.05$), the same occurring with CG patients when compared to controls (C1).

The *XRCC1* and *XRCC3* genotypes and the allele frequency distributions among cases and controls are presented in Table 1. The allele frequencies of polymorphisms 194Trp, 399Gln and 241Met were similar in cases and controls, not showing any statistically significant

Table 1 *XRCC1* and *XRCC3* allele frequencies in patients with chronic gastritis (CG) and gastric cancer (GC) and in the respective control groups C1 and C2

	Genotypes	CG (*n* = 202) *n* (%)	C1 (*n* = 202) *n* (%)	*P*	GC (*n* = 160) *n* (%)	C2 (*n* = 150) *n* (%)	*P*
XRCC1	Arg/Arg	176 (87.1)	183 (90.6)		140 (87.5)	130 (86.7)	
Codon 194	Arg/Trp	24 (11.9)	19 (9.4)		20 (12.5)	19 (12.7)	
	Trp/Trp	2 (1.0)	0 (0.0)		0 (0.0)	1 (0.6)	
	Arg/Trp+Trp/Trp	26 (12.9)	19 (9.4)	0.2683	20 (12.5)	20 (13.3)	0.8269
	Allele frequency	0.07	0.05		0.06	0.07	
XRCC1	Arg/Arg	98 (48.5)	95 (47)		73 (45.6)	70 (46.7)	
Codon 399	Arg/Gln	91 (45.0)	82 (40.6)		67 (41.9)	57 (38.0)	
	Gln/Gln	13 (6.5)	25 (12.4)	0.7651	20 (12.5)	23 (15.3)	0.5035
	Arg/Gln+Gln/Gln	104 (51.5)	107 (53.0)		87 (54.4)	80 (53.3)	
	Allele frequency	0.29	0.33		0.33	0.34	
XRCC3	Thr/Thr	92 (45.5)	84 (41.6)		84 (52.5)	67 (44.7)	
Codon 241	Thr/Met	81 (40.1)	89 (44.1)		53 (33.1)	60 (40.0)	
	Met/Met	29 (14.4)	29 (14.3)	0.4221	23 (14.4)	23 (15.3)	0.1679
	Thr/Met+Met/Met	110 (54.5)	118 (58.4)		76 (47.5)	83 (55.3)	
	Allele frequency	0.34	0.36		0.31	0.35	

Table 2 Association between *XRCC1* and *XRCC3* genotype profiles and risk for chronic gastritis (CG) and gastric cancer (GC)

XRCC1		*XRCC3*										Groups			
Codon 194	Codon 399	Codon 241	CG	GC	OR (95%CI)	*P*	CG	C1	OR (95%CI)	*P*	GC	C2	OR (95%CI)	*P*
All wide-type genotypes														
Arg	Arg	Thr	33	32	1.0 (reference)		33	34	1.0 (reference)		32	23	1.0 (reference)	
One variant polymorphism														
Trp	Arg	Thr	5	8	0.6 (0.17-2.04)	0.5469	5	6	1.2 (0.32-4.16)	1.0000	8	6	1.1 (0.32-3.44)	1.0000
Arg	Gln	Thr	51	38	1.4 (0.68-2.50)	0.5124	51	42	0.8 (0.42-1.51)	0.5234	38	34	1.2 (0.61-2.56)	0.5918
Arg	Arg	Met	50	27	2.0 (0.91-3.57)	0.1237	50	49	1.0 (0.51-1.75)	1.0000	27	34	2,0 (0.83-3.70)	0.1426
Two variant polymorphisms														
Trp	Gln	Thr	3	5	0.6 (0.13-2.63)	0.7106	3	2	0.6 (0.1-4.16)	1.0000	5	4	1.1 (0.16-4.54)	1.0000
Arg	Gln	Met	43	43	1.0 (0.50-1.85)	1.0000	43	58	1.4 (0.69-2.43)	0.4309	43	39	1.2 (0.63-2.50)	0.5389
Trp	Arg	Met	10	6	1.6 (0.52-5.00)	0.5771	10	6	0.6 (0.19-1.78)	0.4106	6	7	1.6 (0.48-5.55)	0.5999
Three variant polymorphisms														
Trp	Gln	Met	8	1	8.3 (0.91-100)	0.0372	8	5	0.6 (0.17-2.04)	0.5400	1	3	5.0 (0.40-50.0)	0.3113

differences ($P>0.05$). However, a combined analysis of the *XRCC1* Arg194Trp and Arg399Gln polymorphisms and the *XRCC3* Thr241Met polymorphism (Table 2), and the assessment of the inter- and intra-gene interactions of these three polymorphisms revealed a statistically significant ($P = 0.0372$) association when the three variant alleles interacted in the chronic gastritis group, as compared to the gastric cancer group. Other combinations did not show any significant difference. The banding patterns of *XRCC1* Arg194Trp and Arg399Gln and of *XRCC3* Thr241Met are represented in Figure 1.

Table 3 shows the associations of the different genotypes with the variables gender, smoking, drinking and *H pylori* infection, among the groups. For this analysis, we combined the heterozygous and mutant homozygous genotypes. Allele 194Trp was associated with smoking, with an increased OR for chronic gastritis (4.16, 95%CI = 1.16-16.66), when we compared the CG and C1 groups. The comparison between the CG and GC groups revealed that in men who were smokers and drinkers there was an association with increased OR's for gastric cancer in the individuals with the alleles *XRCC1* 399Gln (3.03,

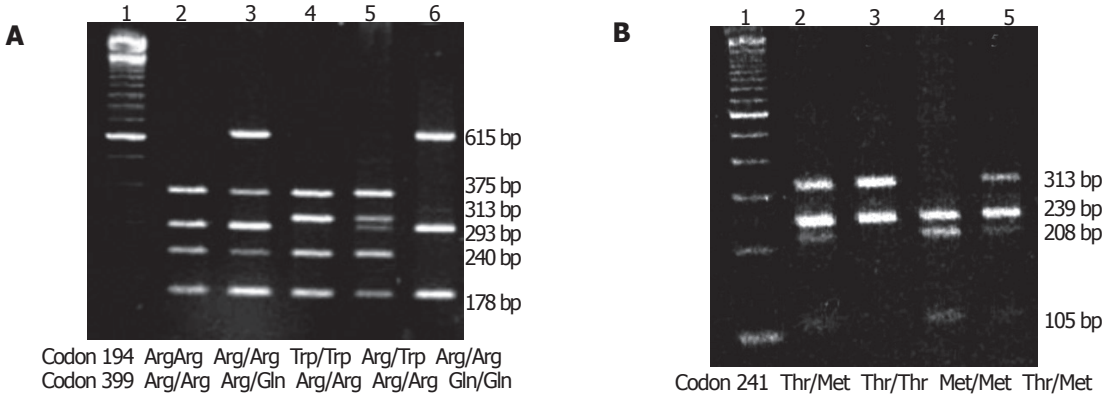

Figure 1 PCR-RFLP of XRCC1 and XRCC3 genes. **A:** *XRCC1* gene. Lane 1: molecular weight marker. Lane 2: wild-type homozygous codons 194 and 399. Lane 3: wild-type homozygous codon 194 and heterozygous codon 399. Lane 4: mutant homozygous codon 194 and wild-type homozygous codon 399. Lane 5: heterozygous codon 194 and wild type-homozygous codon 399. Lane 6: wild-type homozygous codon 194 and mutant homozygous codon 399; **B:** *XRCC3* gene. Lane 1: molecular weight marker. Lanes 2 and 5: heterozygous codon 241. Lane 3: wild-type homozygous codon 241. Lane 4: mutant homozygous codon 241.

Table 3 Association of heterozygous and mutant homozygous to the polymorphisms of codons 194 and 399 of the *XRCC1* gene and to the codon 241 of the *XRCC3* gene with demographic and environmental risk factors in chronic gastritis (CG) and gastric cancer (GC) patients and the respective control groups C1 and C2

	Groups	Variable							
		Gender		Smoke		Drink		*H pylori* infection	
		Male	Female	No	Yes	No	Yes	Positive	Negative
		n (%)	*n* (%)	*n* (%)	*n* (%)	*n* (%)	*n* (%)	*n* (%)	*n* (%)
XRCC1	CG	11 (42.3)	15 (57.7)	9 (34.6)	17 (65.4)	20 (77)	6 (23)		
Arg194Trp									
+	C1	10 (52.6)	9 (47.4)	13 (68.4)	6 (31.6)	17 (89.5)	2 (10.5)		
Trp194Trp		$P = 0.4929$		$P = 0.0250$		$P = 0.4355$			
XRCC1	CG	55 (53)	49 (47)	48 (46.2)	56 (53.8)	71 (68.3)	33 (31.7)	45 (53.6)	39 (46.4)
Arg399Gln	GC	67 (77)	20 (23)	24 (27.6)	63 (72.4)	35 (40.2)	52 (59.8)	9 (23.7)	29 (76.3)
+		$P = 0.0005$		$P = 0.0084$		$P = 0.0001$		$P = 0.0021$	
Gln399Gln	CG	55 (52.9)	49 (47.1)	48 (46.2)	56 (53.8)	71 (68.3)	33 (31.7)		
	C1	52 (48.5)	55 (51.4)	68 (63.6)	39 (36.4)	91 (85)	16 (15)		
		$P = 0.5335$		$P = 0.0111$		$P = 0.0039$			
	GC	67 (77)	20 (23)	24 (27.6)	63 (72.4)	35 (40.2)	52 (59.8)		
	C2	56 (70)	24 (30)	46 (57.5)	34 (42.5)	63 (78.8)	17 (21.2)		
		$P = 0.3042$		$P = 0.0001$		$P = 0.0000$			
XRCC3	CG	57 (51.8)	53 (48.2)	46 (41.8)	64 (58.2)	77 (70)	33 (30)	44 (52.4)	40 (47.6)
Thr241Met	GC	59 (77.6)	17 (22.4)	21 (27.6)	55 (72.4)	36 (47.4)	40 (52.6)	10 (28.6)	25 (71.4)
+		$P = 0.0004$		$P = 0.0494$		$P = 0.0019$		$P = 0.0175$	
Met241Met	CG	57 (51.8)	53 (48.2)	46 (41.8)	64 (58.2)	77 (47.4)	33 (52.6)		
	C1	59 (50)	59 (50)	75 (63.6)	43 (36.4)	98 (79.6)	20 (20.4)		
		$P = 0.7838$		$P = 0.0010$		$P = 0.0110$			
	GC	59 (77.6)	17 (22.4)	21 (27.6)	55 (72.4)	36 (47.4)	40 (52.6)		
	C2	59 (71.1)	24 (28.9)	42 (50.6)	41 (49.4)	66 (79.6)	17 (20.5)		
		$P = 0.3458$		$P = 0.0031$		$P = 0.0000$			

95%CI = 1.58-5.55; 2.27, 95%CI = 1.22-4.16 and 3.22, 95%CI = 1.75-5.88, respectively) and *XRCC3* 241Met (3.22, 95%CI = 1.66-6.25; 1.88, 95%CI = 1.01-3.57 and 2.63, 95%CI = 1.41-4.76, respectively). Comparing the CG and GC groups further, we found increased OR' s for gastric cancer in the association between *H pylori*-negative subjects and the alleles *XRCC1* 399Gln (0.26, 95%CI = 0.11-0.63) and *XRCC3* 241Met (0.36, 95%CI = 0.15-0.85). An association between smoking and drinking and the polymorphisms 399Gln (2.04, 95%CI = 1.17-3.57 and 2.63, 95%CI = 1.35-5.26, respectively) and 241Met (2.44, 95%CI = 1.42-4.16 and 2.13, 95%CI = 1.10-4.00, respectively) was observed when we compared the CG and C1 groups, with increased OR' s for chronic gastritis. Likewise, when we compared GC and C2, we observed an association between smoking and drinking, with an increased OR for stomach cancer, and alleles 399Gln (3.57, 95%CI = 1.85-6.66 and 5.55, 95%CI = 2.77-10.99) and 241Met (2.70, 95%CI = 1.38-5.26 and 4.34, 95%CI = 2.17-9.09). The other evaluated parameters as age and ethnicity, presented no association with the studied polymorphisms.

DISCUSSION

There is increasing evidence that genetic variation leads to different DNA repair capacities in the human population. So, common polymorphisms can play a role in the individual genetic susceptibility to cancer[43]. Very few studies have investigated the role of polymorphisms of the DNA repair genes *XRCC1* and *XRCC3* in the risk of gastric cancer, and, to our knowledge, so far no study examined both gene polymorphisms in this type of cancer and in chronic gastritis. We conducted the first case-control study to investigate the relationship between the polymorphisms *XRCC1* Arg194Trp and Arg399Gln and *XRCC3* Thr241Met and the risk of chronic gastritis and gastric cancer, in a Southeastern Brazilian population.

Our data showed no association between the *XRCC1* and *XRCC3* polymorphisms and an increased risk for chronic gastritis and gastric cancer. To date, there are three published reports on investigations of the association between *XRCC1* polymorphisms and gastric cancer risk[13,27,31], and only one that examined the influence of an *XRCC3* polymorphism[29], with conflicting results. While Shen *et al.*[13] found the wild-genotype Arg194Arg and the mutant-genotype Arg399Gln in the *XRCC1* gene to be associated with an increased risk of gastric cardia cancer, Ratnasinghe *et al.*[27] observed a significant reduction in the risk of this type of cancer, both in Chinese populations. Similarly, Lee *et al.*[31] did not find any association with the risk of gastric cancer in a Korean population, but suggested that the 194Trp allele might be a protective allele with regard to gastric antral cancer. The only study that investigated the role of the *XRCC3* polymorphism in gastric cancer in a Chinese population found no evidence of an association between this polymorphism and an increased risk of gastric cancer[29].

The differences observed in these reports may be due to the different types of gastric cancer studied (cardia and antrum), which may have a distinct pathogenesis and ethnical differences[31]. In the Brazilian population, most gastric cancers are located in the antral region, as in the Korean population, and are commonly associated with H. pylori infection. We also found that in the gastric cancer group the variant allele frequency of 194Trp was lower (0.06) and those of 399Gln and 241Met were higher (0.33 and 0.31, respectively) than those reported in Asian popula tions[13,27,29,31].

These differences can yet be due to the presence of variants of the common susceptibility polymorphisms, not just a single one, but DNA repair genes or activation and detoxification genes may jointly contribute to the susceptibility of gastric and other cancers. Thus, it is important to include more gene polymorphisms for the same or other DNA repair pathways to verify the gene-gene interactions, as well as the gene-environment interactions that may be important in the etiology of the disease.

When we assessed the association of *XRCC1* Arg194trp and Arg399Gln and *XRCC3* Thr241Met with the risk of chronic gastritis and gastric cancer, we found an increased risk for chronic gastritis when all three variant alleles were present at the same time, supporting the hypothesis of an additive effect of these three polymorphisms. There are no studies on chronic gastritis regarding its association with DNA repair gene polymorphisms, as there are for several metabolizing genes, such as *GSTM1*, *GSTT1* and *CYP2E1*, in Brazilian[44] and Chinese[45] populations. Chronic gastritis, a frequent inflammation of the stomach[46], is considered a premalignant lesion[47]. Gastritis may start after an *H pylori* infection and progress over time from an initially superficial form to more severe forms, including severe atrophic gastritis with intestinal metaplasia[48]. About 10% of patients with gastric atrophy develop gastric cancer within a time period of 15 years[46]. Therefore, a reduced DNA repair capacity due to variant alleles may allow mutations to accumulate in the DNA of the epithelial cells of the stomach, resulting from the inflammatory process caused by *H pylori* or from environmental factors such as dietary habits and lifestyle, increasing the risk of gastric cancer.

Gastric cancer has a complex etiology in which genetic and environmental factors play an important role. In this study, we observed a statistically significant association between variant alleles and demographic and environmental factors such as gender, smoking, drinking and *H pylori* infection.

It is known that *H pylori* infection has a very important role in the development of chronic gastritis and in its development into gastric cancer[48]. Various mechanisms were proposed for *H pylori*-associated carcinogenesis, such as the formation of DNA adducts, the generation of free radicals, and a dysregulation of the gastric epithelial cell cycle[49,50]. So, these factors, associated with a decreased DNA repair capacity, may increase the risk for gastric cancer. Differently from these findings, we found a high frequency of variants 399Gln and 241Met

in *H pylori*-negative gastric cancer patients, as compared to chronic gastritis patients. However, these data must have been influenced by the great number of *H pylori*-negative individuals, as compared to the H. pylori-positive individuals found in the gastric cancer group, probably due to an underestimate of the histological diagnosis in these patients.

In smokers, the presence of *XRCC1* 194Trp was more frequent in chronic gastritis cases, while polymorphisms *XRCC1* 399Gln and *XRCC3* 241Met were more frequent in both the chronic gastritis and the gastric cancer groups, compared with healthy controls. Whether the mechanisms of tobacco carcinogens act in human gastric cancer is currently uncertain. The main carcinogens contained in tobacco smoke include polyaromatic hydrocarbons (PAH), *N*-nitrosamines and aromatic amines. Cigarette smoking increases the number of single-strand breaks and DNA adducts, which, if left unrepaired, can lead to gene mutation[51]. These DNA damages can be repaired by BER, in which the *XRCC1* protein has an important role. Functional studies of *XRCC1* variants observed a significantly elevated level of sister chromatid exchange (SCE) in peripheral blood lymphocytes after *in vitro* exposure to the tobacco-specific NNK in carriers of the 399Gln allele, but the same was not observed for the 194Trp polymorphism[52]. Duell *et al*.[53] reported higher frequencies of SCE for current smokers with the 399Gln polymorphism than for smokers with the Arg/Arg genotype.

Protein *XRCC3* participates in the DSB repair by the homologous repair pathway and the 241Met variant may lead to biological implications for the enzyme's function and/or the interaction with other proteins involved in DNA damage repair. Matullo *et al*.[54] associated the 241Met polymorphism with ^{32}P-DNA adduct levels, indicating a possible role of the *XRCC3* gene in the repair of bulky DNA adducts. Thus, variations in DNA repair capacity caused by polymorphisms of DNA repair genes may modulate the genotoxic effect of tobacco smoking.

Excessive alcohol consumption can also lead to DNA damage through the production of free radical intermediates, such as reactive oxygen species, which are produced during the ethanol metabolism[55]. The frequency of DNA single-strand breaks also increases with chronic exposure to alcohol[56]. We observed an association with alcohol consumption in the patients with chronic gastritis and gastric cancer, and found an increased risk for these diseases when polymorphisms *XRCC1* 399Gln and *XRCC3* 241Met were present.

In conclusion, in the Brazilian population studied, we did not find evidence of a relationship between the polymorphisms *XRCC1* Arg194Trp and Arg399Gln and *XRCC3* Thr241Met and the development of chronic gastritis and gastric cancer. However, intra- and inter-gene interactions may contribute to the development of chronic gastritis, a precursor lesion of stomach cancer. We also verified a gene-environment interaction between the *XRCC1* and *XRCC3* polymorphisms, mainly with the habits of smoking and drinking, in the chronic gastritis and gastric cancer patients. Our study is an important addition to the small number of previously published reports on DNA repair gene variants in gastric cancer and shows the need for further studies in different populations, to elucidate the role of these polymorphisms in carcinogenesis.

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Expression and significance of CD44s, CD44v6, and nm23 mRNA in human cancer

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Abstract

AIM: To investigate the relationship between the expression levels of nm23 mRNA, CD44s, and CD44v6, and oncogenesis, development and metastasis of human gastric adenocarcinoma, colorectal adenocarcinoma, intraductal carcinoma of breast, and lung cancer.

METHODS: Using tissue microarray by immu-histochemical (IHC) staining and *in situ* hybri-dization (ISH), we examined the expression levels of nm23 mRNA, CD44s, and CD44v6 in 62 specimens of human gastric adenocarcinoma and 62 specimens of colorectal adenocarcinoma; the expression of CD44s and CD44v6 in 120 specimens of intraductal carcinoma of breast and 20 specimens of normal breast tissue; the expression of nm23 mRNA in 72 specimens of human lung cancer and 23 specimens of normal tissue adjacent to cancer.

RESULTS: The expression of nm23 mRNA in the tissues of gastric and colorectal adenocarcinoma was not significantly different from that in the normal tissues adjacent to cancer ($P>0.05$), and was not associated with the invasion of tumor and the pathology grade of adenocarcinoma ($P>0.05$). However, the expression of nm23 mRNA was correlated negatively to the lymph node metastasis of gastric and colorectal adenocarcinoma ($r = -0.49$, $P<0.01$; $r = -4.93$, $P<0.01$). The expression of CD44s in the tissues of gastric and colorectal adenocarcinoma was significantly different from that in the normal tissues adjacent to cancer ($P<0.05$; $P<0.01$). CD44v6 was expressed in the tissues of gastric and colorectal adenocarcinoma only, the expression of CD44v6 was significantly associated with the lymph node metastasis, invasion and pathological grade of the tumor ($r = 0.47$, $P<0.01$; $r = 5.04$, $P<0.01$). CD44s and CD44v6 were expressed in intraductal carcinoma of breast, the expression of CD44s and CD44v6 was significantly associated with lymph node metastases and invasion ($P<0.01$). However, neither of them was expressed in the normal breast tissue. In addition, the expression of CD44v6 was closely related to the degree of cell differentiation of intraductal carcinoma of breast ($\chi^2 = 5.68$, $P<0.05$). The expressional level of nm23 mRNA was closely related to the degree of cell differentiation ($P<0.05$) and lymph node metastasis ($P<0.01$), but the expression of nm23 gene was not related to sex, age, and type of histological classification ($P>0.05$).

CONCLUSION: Patients with overexpression of CD44s and CD44v6 and low expression of nm23 mRNA have a higher lymph node metastatic rate and invasion. In addition, overexpression of CD44v6 is closely related to the degree of cell differentiation. Detection of the three genes is able to provide a reliable index to evaluate the invasion and metastasis of tumor cells.

Key words: Tissue microarray; Nm23 mRNA; CD44s; CD44v6; Gastric adenocarcinoma; Colorectal adeno-carcinoma; Intraductal carcinoma of breast; Lung cancer

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INTRODUCTION

Tissue microarray (TMA) technology is a new method used to analyze hundreds of tumor samples on a single slide, allowing high resolution analysis of genes and proteins on a large cohort. TMA is ideally suitable for genomics-based diagnosis and drug target locating. Oncogenesis, development and metastasis of tumor are triggered by many genes and factors. In recent years, research on the molecular mechanism of oncogenesis and metastasis and the diagnosis of the correlated mark of tumor are

important objects in clinical research in oncology. CD44 is one of the transmembrane proteins on the surface of cells. Its distribution is very extensive and can be detected in lymphocytes and fibroblasts[1]. The expression of CD44v, which is related to the progression, metastasis and prognosis of tumor, has been detected in lung cancer, carcinoma of colon, esophageal cancer, carcinoma of breast, carcinoma of urinary bladder, liver cancer, cervix cancer, carcinoma of kidney and reticulosarcoma[2-5]. Nm23 was first found by Steeg in 1988. Lower expression of nm23 is related to the metastasis of tumors, such as carcinoma of breast, lung cancer, gastric carcinoma, malignant melanoma, and ovary cancer, and has been regarded as the gene of transfer inhibition. In order to investigate the relationship between the expression level of nm23 mRNA, CD44s and CD44v6 and the oncogenesis, pathological grade, invasion, and metastasis of tumor, we detected the expression of nm23 mRNA, CD44s, and CD44v6 in tissues of human gastric adenocarcinoma, colorectal adenocarcinoma, intraductal carcinoma of breast and lung cancer, and also in tissues adjacent to cancer using tissue microarray by immunohistochemical (IHC) staining and *in situ* hybridization (ISH).

MATERIALS AND METHODS

Tissue specimens

The following tissue specimens were enrolled in this study: 40 specimens of colorectal adenocarcinoma (including 16 specimens of moderately differentiated colorectal adenocarcinoma, 24 poorly differentiated adenocarcinoma, 14 lymph node metastatic carcinoma, and 17 invasive carcinoma) and 22 specimens of normal colorectal tissue, 40 specimens of gastric adenocarcinoma (including 20 specimens of moderately differentiated gastric adenocarcinoma, 20 poorly differentiated adenocarcinoma, 15 lymph node metastatic carcinoma, and 23 invasive carcinoma) and 22 specimens of normal gastric tissue, 120 specimens of intraductal carcinoma of breast (including 86 specimens of moderately differentiated intraductal carcinoma, 34 poorly differentiated intraductal carcinoma, 30 lymph node metastatic carcinoma and 58 invasive carcinoma) and 20 specimens of normal breast tissue, 72 specimens of lung cancer (including 13 specimens of moderately differentiated carcinoma, 19 poorly differentiated carcinoma, 24 lymph node metastatic carcinoma) and 23 specimens of normal lung tissue. All patients underwent surgery at Xijing Hospital of the Fourth Military University, China in 2003. The resected specimens were fixed in 10% formaldehyde.

Reagents

CD44s and CD44v6 monoclonal antibodies were obtained from MBI. SABC kit was bought from Sina-America Biotechnology Company. nm23 oligonucleotide probe and CSA test kit were purchased from Boshide Biotechnology Company. Biotin labeled anti-digitoxin (DIG) antibody was obtained from Sigma.

Tissue microarray

Samples were fixed in buffered formaldehyde and subsequently paraffin-embedded. Histological sections (5 μm) were prepared from the specimens, and then diagnosed and labeled by the pathologist. Tissue microarray was performed on the instrument of microarray from Beecher, USA. A hole (diameter 0.5 mm) was made on the recipient block (blank paraffin block), then the tissue core was taken on the supply block (labeled tissue block) and put in the hole. The former procedure was repeated and tissue microarray was completed. Histological sections (3-5 μm) were prepared with the instrument of Leica, Germany, and then rediagnosed by the pathologist.

Immunohistochemical analysis

The sections for tissue microarray were dewaxed in water by normal technique, and then the antigen was restored in high pressure (at 121 °C for 10 min). Immunohistochemistry was performed with the SABC kit. The primary antibodies were CD44s and CD44v6 monoclonal antibodies. Staining was performed following the instructions of SABC kit.

Detection of nm23 mRNA expression by in situ hybridization

All reagents and containers were treated by diethylprocarbonate (DEPC). In brief, the sections for tissue microarray were dewaxed in water by normal technique, immersed into 3% H_2O_2 at room temperature for 10 min, and then washed twice with distilled water. Twenty microgram per milliliter of freshly diluted protease K was added and digested for 20 min at 37 °C to expose mRNA nucleic acid segments. Twenty milliliter of glycerine (20%) was added to the dry bottom of the test kits to keep humidity, 20 μL of the pre-hybridization solution was added to each section and kept at 37 °C for 4 h. Then the supernumerary liquid was absorbed without washing, hybridization solution was added as mentioned above. The sections were then covered with protective membrane and put in homeothermia at 40 °C overnight. After the coverglass was removed, the sections were washed twice at 37 °C in 2×SSC for 5 min, once in 0.5×SSC for 15 min, once in 0.2×SSC for 15 min, and kept in 3% BSA at 37 °C for 30 min and in seal solution at 37 °C for 30 min. After being washed with 0.5 mol/L PBS, the biotin labeled anti-digitoxin antibody was added for 60 min at 37 °C, followed by biotin-peroxidase for 20 min at 37 °C and then washed with PBS. Color was showed by DAB. Finally, the sections were restained, dehydrated, pellucidated, and sealed. Negative controls were designed.

Assessment of immunohistochemical staining and in situ hybridization

Specimens were considered positive when >50% of the tissue components were immunohistochemically stained brown-yellow in appropriate cellular compartment. Specimens were considered positive for ISH when >50% of the tissue components were stained blue in appropriate cellular compartment.

Statistical analysis

Statistical analysis was performed with χ^2 test. $P<0.05$ was considered statistically significant.

RESULTS

Relationship between expression of CD44s, CD44v6, nm23 mRNA, and clinical pathology of human colorectal adenocarcinoma

The expression of CD44s in tissues of colorectal adenocarcinoma and normal colorectal mucosa was 42.0% (17/40) and 13.6% (3/22), respectively (Figures 1A and B), and there was a significant difference between them ($\chi^2 = 5.18$, $P<0.05$). In addition, there was a statistical significance between colorectal adenocarcinoma of with and without invasion ($\chi^2 = 10.52$, $P<0.01$), as well as with and without lymph node metastasis ($\chi^2 = 12.48$, $P<0.01$). However, there was no statistical significance between moderately and poorly differentiated colorectal adenocarcinoma ($P>0.05$), indicating that the expression of CD44s was related to the oncogenesis and development of colorectal adenocarcinoma. The expression of CD44v6 in tissues of colorectal adenocarcinoma and normal colorectal mucosa was 55.8% (22/40) and 0% (0/22), respectively (Figure 1C). There was a statistical significance between moderately and poorly differentiated colorectal adenocarcinoma ($P<0.05$) with and without invasion ($P<0.05$), as well as with and without lymph node metastasis ($\chi^2 = 9.22$, $P<0.01$). The results showed that the expression of CD44v6 was related not only to the invasion of colorectal carcinoma, but also to the metastasis. The expression of nm23 mRNA in tissues of colorectal adenocarcinoma and normal colorectal mucosa was 45.0% (18/40) and 40.9% (9/22), respectively (Figure 1D), and there was no statistically significant difference ($P>0.05$). However, the expression of nm23 mRNA in gastric adenocarcinoma with/without lymph node metastasis was 28.5% (4/14) and 53.8% (14/26), respectively. The difference was statistically significant ($\chi^2 = 8.47$, $P<0.05$). In addition, the expression of CD44v6 in colorectal adenocarcinoma was related to the invasion ($P<0.05$), but not to the pathological grade ($P>0.05$, Table 1).

Relationship between expression of CD44s, CD44v6, and nm23 mRNA in human colorectal adenocarcinoma

Expression of CD44s and CD44v6 was associated with invasion and lymph node metastasis of colorectal adenocarcinoma ($r = 0.47$, $P<0.05$). However, expression of nm23 mRNA was not associated with invasion and lymph node metastasis of colorectal adenocarcinoma ($r = -0.49$, $P<0.05$), suggesting that CD44s, CD44v6 and nm23 mRNA could regulate invasion and lymph node metastasis of colorectal adenocarcinoma. In addition, the expression level of nm23 mRNA, CD44s and CD44v6 was not associated with the age and sex of patients ($P>0.05$, Table 1).

Relationship between expression of CD44s, CD44v6, nm23 mRNA, and clinical pathology of human gastric adenocarcinoma

The expression of CD44s in tissues of gastric adenocarcinoma and normal gastric mucosa was 48% (19/40) and 13.6% (3/22), respectively (Figures 2A and B), and there was a significant difference between them ($\chi^2 = 10.29$, $P<0.01$). However, the expression of CD44s was not significantly associated with lymph node metastasis. There was no statistical significance between moderately and poorly differentiated gastric

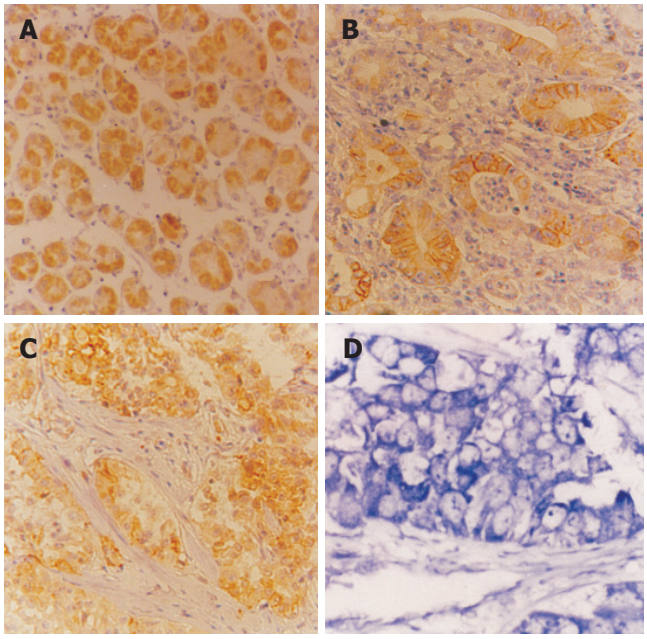

Figure 1 Expression of CD44s in human normal colorectal and adenocarcinoma tissue (**A**, **B**) and expression of CD44v6 (**C**) and nm23 mRNA (**D**) in human colorectal adenocarcinoma.

Table 1 Relationship between expression of CD44s, CD44v6, nm23 mRNA and pathologic feature of human colorectal adenocarcinoma

Pathologic feature	*n*	CD44s Positive (%)	CD44s *P*	CD44v6 Positive (%)	CD44v6 *P*	nm23 mRNA Positive (%)	nm23 mRNA *P*
Grade			>0.05		<0.05[a]		>0.05
II	16	7(43.7)		11(68.7)		8(50.0)	
I	24	10(41.6)		11(45.8)		10(41.6)	
Invasion			<0.01[b]		<0.01[b]		<0.05[c]
+	17	11(64.7)		12(70.5)		5(29.4)	
–	23	6(26)		10(43.4)		13(56.5)	
Metastasis			<0.0[d]		<0.05[e]		<0.05[e]
+	14	9(64.2)		10(71.4)		4(28.5)	
–	26	8(30.7)		12(46.1)		4(53.8)	
Age/yr			>0.05		>0.05		>0.05
>60	19	8(42.1)		10(52.6)		8(42.1)	
<60	21	9(42.8)		12(57.1)		10(47.6)	
Sex			>0.05		>0.05		>0.05
M	26	10(38.4)		14(53.8)		12(46.1)	
F	14	7(50)		8(57.1)		6(42.8)	
Normal	22	4(18.1)		0(0)		9(40.9)	

[a]$P<0.05$ *vs* poorly differentiated colorectal adenocarcinoma; [c]$P<0.05$, [b]$P<0.01$ *vs* without invasion; [e]$P<0.05$, [d]$P<0.01$ *vs* without metastasis.

adenocarcinoma with/without invasion (P>0.05). The expression of CD44v6 in tissues of gastric adenocarcinoma and normal gastric mucosa was 63.3% (25/40) and 0% (0/22), respectively (Figure 2C). There was a statistical significance between moderately and poorly differentiated gastric adenocarcinoma (χ^2 = 9.19, P<0.01) with and without invasion (χ^2 = 22.22, P<0.01), as well as with and without lymph node metastasis (χ^2 = 10.36, P<0.01). The expression of nm23 mRNA in tissues of gastric adenocarcinoma and normal gastric mucosa was 47% (19/40) and 43% (9/22), respectively (Figure 2D) and there was no statistically significant difference between them (P>0.05). However, the expression of nm23 mRNA in gastric adenocarcinoma with/without lymph node metastasis was 26.7% (4/15) and 60% (15/25) respectively, the difference was significant (χ^2 = 18.47, P<0.01, Table 2).

Relationship between expression of CD44v6 and nm23mRNA in human gastric adenocarcinoma

The expression of CD44v6 was associated with lymph node metastasis of gastric adenocarcinoma (r = 5.04). However, the expression of nm23 mRNA was not associated with lymph node metastasis of gastric adenocarcinoma (r = -4.93, Table 2).

Relationship between the expression of CD44s, CD44v6, and clinical pathology of human intraductal carcinoma of breast

The expression of CD44s and CD44v6 in tissues of intraductal carcinoma of breast was 45.8% (55/120) and 53.3% (64/120), respectively (Figures 3A and B), but neither of them was expressed in normal breast tissue. The expression of CD44s and CD44v6 in intraductal breast carcinoma with and without invasion had a significant statistical difference (χ^2 = 9.52, P<0.01; χ^2 = 22.89, P<0.001), as well as with and without lymph node metastasis (χ^2 = 9.41, P<0.01; χ^2 = 8.75, P<0.01). Furthermore, the expression of CD44s was not significantly associated with moderately and poorly differentiated intraductal breast carcinoma (P>0.05). However, the expression of CD44v6 was not only related to invasion and lymph node metastasis of intraductal breast carcinoma, but also related to its pathological grade (χ^2 = 5.68, P<0.05, Table 3).

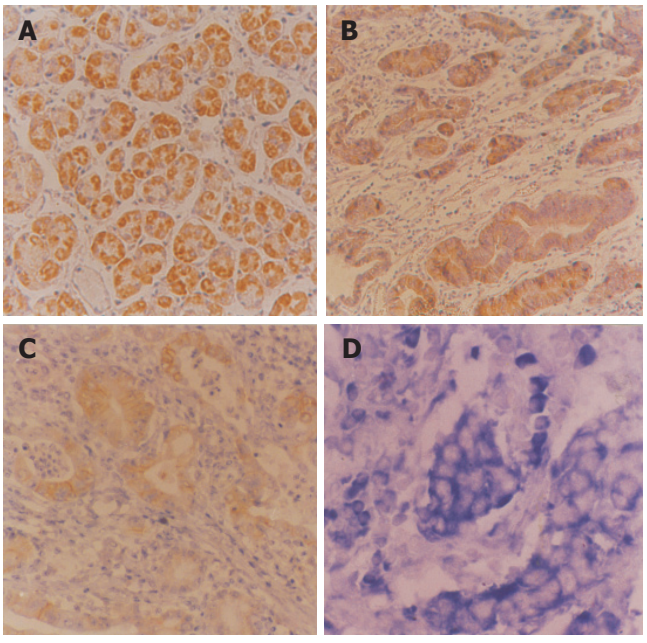

Figure 2 Expression of CD44s in human normal gastric tissue (**A, B**) and expression CD44v6 (**C**) and nm23 mRNA (**D**) in human gastric adenocarcinoma

Relationship between expression of nm23 mRNA and clinical pathology of human lung cancer

The expression of nm23 mRNA in tissues of lung cancer and normal lung tissues was 55.2% (40/72) and 82.6% (19/23), respectively (Figure 4), and there was a significant difference between them (χ^2 = 5.42, P<0.05). The expression of nm23 mRNA in tissues of lung cancer with and without lymph node metastasis was 25.0% (6/24) and 70.8% (34/48), respectively and there was a significant difference between them (χ^2 = 13.61, P<0.001), as well as between moderately and poorly differentiated lung cancer (χ^2 = 9.61, P<0.01), indicating that the expression of nm23 mRNA was associated with the oncogenesis, lymph node metastasis and pathological grade of lung cancer. In addition, the expression of nm23 mRNA in lung cancer was not associated with the age and sex of patients and the pathologic feature of lung cancer (P>0.05, Table 4).

DISCUSSION

CD44s, a hyaluronic acid receptor, is important in regulating invasion and metastasis of tumor[3-7]. Previous studies demonstrated that the expression of CD44s is an important biological marker for predicting metastatic potential. Invasion and metastasis of tumor are a very complicated process, which is regulated by many correlated genes. CD44v6 may take part in the invasion and meta-

Table 2 Relationship between expression of CD44s, CD44v6, nm23 mRNA, and pathologic feature of human gastric adenocarcinoma

Pathologic feature	n	CD44s			CD44v6			nm23 mRNA		
		Positive	%	P	Positive	%	P	Positive	%	P
Grade				>0.05			[b]<0.01			>0.05
II	20	8	40.0		10	50.0		9	45.0	
III	20	10	50.0		15	75.0		10	50.0	
Invasion				>0.05			[d]<0.01			>0.05
+	23	11	47.8		18	78.2		11	47.8	
-	17	7	41.1		7	41.0		8	47.1	
Metastasis				>0.05			[f]<0.01			[f]<0.01
+	15	6	40.0		12	80.0		4	26.7	
-	25	12	48.0		13	52.0		15	60.0	
Age/year				>0.05			>0.05			>0.05
>60	21	10	47.2		13	61.9		9	42.9	
<60	19	8	42.1		12	63.1		10	52.6	
Normal	22	3	13.6		0	0		9	43.0	

[b]P <0.01 *vs* well-differentiated gastric adenocarcinoma; [d]P <0.01 *vs* without invasion; [f]P <0.01 *vs* without metastasis.

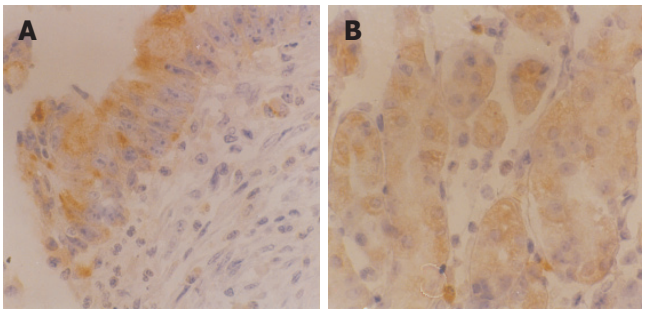

Figure 3 Expression of CD44s (**A**) and CD44v6 (**B**) in human intraductal breast carcinoma.

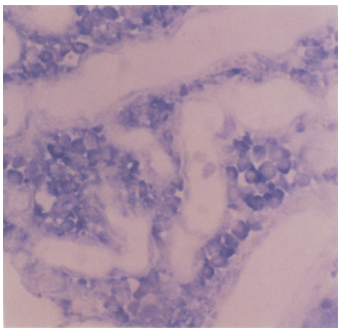

Figure 4 Expression of nm23 mRNA in human lung cancer.

Table 3 Relationship between the expression of CD44s and CD44v6 and pathologic feature of human intraductal breast carcinoma

Pathologic features	*n*	CD44s			CD44v6		
		Positive	%	*P*	Positive	%	*P*
Grade				>0.05			<0.05[a]
II	86	38	44.2		40	46.5	
III	34	17	50		24	70.1	
Invasion				<0.01[b]			<0.01[b]
+	58	35	60.3		44	75.9	
−	62	20	32.3		20	32.3	
Metastasis				<0.01[d]			<0.01[d]
+	30	21	70		23	76.7	
−	90	34	37.8		41	45.6	
Age(yr)				>0.05			>0.05
>50	66	31	47.1		34	51.5	
<50	54	24	44.4		30	55.6	
Normal	20	0	0		0	0	

[a]*P*<0.05 *vs* well-differentiated intraductal breast carcinoma; [b]*P*<0.01 *vs* without invasion; [d]*P*<0.01 *vs* without metastasis.

Table 4 Relationship between expression of nm23 mRNA and pathologic feature of lung cancer

Pathologic feature	*n*	nm23 mRNA		
		Positive	%	*P*
Grade				<0.01[b]
I	19	3	15.8	
II	13	7	53.8	
III	20	17	85	
Metastasis				<0.01[d]
+	24	6	25	
−	48	34	70.8	
Sex				>0.05
M	51	28	54.9	
F	21	12	57.1	
Age(yr)				>0.05
>55	42	22	52.3	
<55	30	18	60	
Normal	23	19	82.6	

[b]*P*<0.01 *vs* moderately and well-differentiated lung cancer; [d]*P*<0.01 *vs* without metastasis.

stasis of tumor, but it is not the necessary factor for tumor invasion and metastasis. When CD44v6 produces a marked effect, it must be restricted by many factors in vivo. It has been proved that CD44v6 is correlated to tumor invasion and metastasis in experiments of cultured cells and animals. But consistent view has not been reached so far with respect to the relationship between expression of CD44v6 and human tumor invasion and metastasis.

It was reported that CD44v mRNA is expressed in human colorectal carcinoma as detected by RT-PCR[8-10]. Zalewski *et al*.[11] reported that the expression of CD44 is associated with the size of tumor, age, and sex of patients. In our current study, CD44 and CD44v6 were expressed in colorectal carcinoma and normal colorectal tissues respectively, indicating that the expression of CD44 and CD44v6 is associated with the invasion and metastasis of colorectal carcinoma, but not associated with the age and sex of colorectal carcinoma patients. In addition, the expression of CD44v6 was related to the pathological grade of tumor. Other studies reported that the expression of CD44 is not associated with the clinical pathological feature of colorectal carcinoma[12,13]. Whether the expression level of CD44 can be seen as a marker of tumor histopathology requires further research.

Using reverse transcription polymerase chain reaction followed by Southern blotting, Yamamichi *et al*.[14] examined the expression of the standard and variant forms (v6 and v9) of CD44 mRNA in 73 cases of gastric cancer, and found that the expression status of the standard form of CD44 mRNA is correlated with peritoneal dissemination only, and that of CD44v9 mRNA does not significantly correlate with any clinicopathologic factor. Li *et al*.[15] reported that 74% gastric cancers and 80% invasive carcinomas are positive for CD44v6, implying that CD44v6 is also a useful marker of tumor invasion and metastasis. In the present study, the expression of CD44v6 was significantly correlated with tumor differentiation, lymph node metastasis and invasion of tumor. The expression of CD44s and CD44v6 in gastric cancer was much higher than that in normal stomach and was associated with genesis, metastasis and clinically aggressive behavior of gastric adenocarcinoma.

The aberrant activation of two or more genes plays a different role during different stages of genesis, progression and metastasis of malignant tumor. These genes synergically facilitate carcinoma change. Our studies showed that the expression of nm23 mRNA in colorectal adenocarcinoma was negatively correlated with that of

CD44 and CD44v6, which was closely associated with the invasion and metastasis of colorectal adenocarcinoma. These results indicate that nm23 and CD44v6 synergically play positive and negative roles during lymph node metastasis of colorectal, and gastric adenocarcinoma. Further studies are needed to investigate the relationship between nm23 and CD44v6, which is important for tumor metastasis.

Mammary cancer is one of the most common malignant tumors. Although 50% of mammary cancers are surgically curable, 50% patients have metastasis within 5 years after surgery. Kaufmann *et al*.[16] reported that the positive expression rate of CD44v6 is 80% in primary tumor and 100% in focal tumor, but no expression is found in normal mammary tissue. The patients with positive expression of CD44v6 have a poor prognosis. There are still many correlative reports[17–20]. Our results showed that the expression of CD44s and CD44v6 in intraductal carcinoma was related with tumor invasion and metastasis, but not with the age and sex of patients.

Since the gene of nm23 was found, lower expression of nm23 has been found to be associated with high metastatic potential and poor prognosis of human mammary cancer, gastric cancer, lung cancer, melanoma, and ovary cancer. Nm23H1 is more important than nm23H2. Gazzeri *et al*.[21] reported that the expression of nm23H1 is not associated with clinicopathological features of adenocarcinoma, while it is associated with the development of squamous carcinoma. Lai *et al*.[22] reported that the expression of nm23H1 is positively correlated with tumor metastasis and prognosis. The lower the nm23H1 expression is, the poorer the prognosis is. Our studies showed that the expression of nm23 mRNA was correlated with lymph node metastasis (r = -4.93). The expression of nm23 mRNA was gradually reduced in normal lung tissue adjacent to cancer. In conclusion, nm23H1 may take part in the regulation of metastasis as a prohibitive gene, and may become a valuable target in the evaluation of tumor development and prognosis.

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Assessment of KL-6 as a tumor marker in patients with hepatocellular carcinoma

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Abstract

AIM: To investigate the clinical significance of KL-6 as a tumor marker of HCC in two different ethnic groups with chronic liver disease consecutively encountered at outpatient clinics.

METHODS: Serum KL-6 was measured by the sandwich enzyme immunoassay method using the KL-6 antibody (Ab) as both the capture and tracer Ab according to the manufacturer's instructions (Eisai, Tokyo, Japan). Assessment of alpha fetoprotein (AFP) and protein induced vitamin K deficiency or absence (PIVKA-II) was performed in both groups using commercially available kits.

RESULTS: A significantly higher mean serum KL-6 (556±467 U/L) was found in HCC in comparison with non-HCC groups either with (391±176 U/L; P<0.001) or without (361±161 U/L; P<0.001) liver cirrhosis (LC). Serum KL-6 level did not correlate with either AFP or PIVKA-II serU/Levels. Using receiver operating curve analysis for KL-6 as a predictor for HCC showed that the area under the curve was 0.574 (95%CI = 0.50-0.64) and the KL-6 level that gave the best sensitivity (61%) was found to be 334 U/L but according to the manufacturer's instructions; a cut-off point of 500 U/L was used that showed the highest specificity (80%) in comparison with AFP and PIVKA-II (78% *vs* 72% respectively). Combining the values of the three markers improved specificity of AFP for HCC diagnosis from 78% for AFP alone; 93% for AFP plus PIVKA-II to 99% for both plus KL-6 value (P<0.001). Mean serum alkaline phosphatase level was significantly higher in KL-6 positive (564±475) in comparison with KL-6 negative (505±469) HCC patients (P = 0.021), but such a difference was not found among non-HCC corresponding groups.

CONCLUSION: KL-6 is suggested as a tumor for HCC. Its positivity may reflect HCC-associated cholestasis and/or local tumor invasion.

Key words: Tumor markers; Liver disease; Hepatocellular carcinoma

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the 4th most common cancer worldwide, and it is a well-known complication of chronic hepatitis[1,2]. Asymptomatic patients diagnosed as HCC through screening programs are more likely to be candidates for curative treatment and have improved short- and medium-term survival[3,4]. Although serum alpha-fetoprotein (AFP) had been shown to be associated with HCC since 1963[5], unfortunately it is also elevated in a wide variety of non-hepatic malignancies[6,7] and benign hepatic conditions[8,9]. Moreover, it is uncertain whether serum AFP is a useful marker for HCV-related HCC in some ethnic groups e.g., North American patients of African origin[10]. Thus, searching another tumor marker, that together with AFP could improve the diagnostic utility of the later, seemed to be justified. KL-6 was originally found using a murine monoclonal antibody that recognized an undefined sialylated carbohydrate chain on a mucin-like glycoprotein[11] which was also defined as MUC1[12]. The cell membrane MUC1 was found to regulate cell adhesion properties[13]. KL-6 has been first shown to be

Table 1 Background data of the study groups

	Egyptian			Japanese		
	HCC (+)	HCC (-)	*P*	HCC (+)	HCC (-)	*P*
	n = 65	*n* = 106		*n* = 45	*n* = 128	
Mean age (SD, yr)	57±11[b]	47±9	<0.001	66±10[b]	63±10	NS
Age <50 yr	16 (25)[d]	65 (61)	<0.001	3 (7)[d]	17 (13)	NS
Male	50 (77)	82 (77)	NS	38 (84)	87 (68)	0.024
Liver disease						
Viral	61 (94)	96 (91)		44 (98)	107 (84)	
HCV-related	59 (91)	92 (87)	NS	36 (80)	81 (63)	0.031
HBV-related	2 (3)[1]	4 (4)	NS	8 (18)[1]	28 (22)	NS
Non-viral	4 (6)	10 (9)	NS	1 (2)	20 (16)	0.010
Cirrhosis	46 (71)	45 (42)	<0.001	40 (89)	40 (31)	<0.001
Child's C	25 (38)[f]	17 (16)	0.001	4 (9)[f]	1 (1)	0.017
Mean±(SD)						
ALT (IU/L)	73±95	66±45	0.08	55±35	50±39	NS
Serum Albumin (g/L)	3.0±0.7	3.0±0.5	NS	3.6 ±0.5	4.2±0.4	<0.001
Platelet count×1 000/mL^3	186±107[h]	89±53	0.001	130±51[h]	170±71	<0.001
AFP >10 ng/mL (+)	64 (99)	28 (26)	<0.001	30 (67)	23 (18)	<0.001
PIVIKA>40 mAU/L (+)	51 (79)	38 (36)	<0.001	16 (36)	27 (21)	0.047

[b]P<0.001, [d]P<0.001, [f]P<0.001, [h]P<0.001 *vs* Japannese, [1]P = 0.001.

elevated in patients with interstitial pneumonia[14]. It was also reported to have a high positive rate in different non-hepatic malignancies and its expression was also correlated with metastatic potential of the primary tumor in some of them[15-17]. It has also been studied as a fibrosis marker in patients with HCV-related chronic liver disease[18] and was found to correlate with the degree of irregular regeneration of hepatocytes[19]. A recent study addressed its clinical significance as a tumor marker in HCV-related HCC[20]. However, all these studies investigated KL-6 in HCV-related disease only so that its actual significance as a marker for screening HCC in patients with different chronic liver disease is not yet fully understood. In this study, we aimed to investigate KL-6 as a tumor marker in consecutive patients with chronic liver disease seen at outpatient settings in two different ethnic groups of possible different risk factors for HCC, so that we could get a wider spectrum of disease in order to assess KL-6 validity for HCC screening.

MATERIALS AND METHODS

Study population

We conducted a cross-sectional study between October 2001 and November 2002. Data were gathered from two Affiliations; Shinshu University (Japan) and Suez Canal University (Egypt) Hospitals. A total of 334 consecutive patients with chronic liver disease seen at outpatient liver clinics in the two settings (who met our inclusion/exclusion criteria) were included; of them: 110 patients were diagnosed as HCC with a mean age of 61±11 years and M:F (4:1). Sixty-five were Egyptians and 45 Japanese with viral-related liver disease accounting for 94% and 98% of them respectively. Non-HCC patients were 234 with a mean age of 56±13 years; M:F (7/3). One hundred and six were Egyptians and 128 Japanese with viral-related liver disease accounting for 91% and 84% of them respectively (Table 1).

Chronic liver disease and cirrhosis were identified and diagnosed according to liver biopsy findings, clinical and/or radiological evidence of portal hypertension. HCC was excluded by imaging studies (abdominal ultrasound (US), computed tomography (CT), magnetic resonance imaging (MRI) and/or hepatic angiography), one of which must have been performed at least 6 months following the measurement of AFP.

HCC was diagnosed when meeting our inclusion criteria of positive cytology and/or histology or by the presence of characteristic hepatic masses on liver CT, MRI and/or hepatic angiography (i.e., enlarging tumors and/or tumors with typical arterial vascularization).

We excluded patients with alcoholic and schistosomal liver diseases from our study populations. We had also excluded patients known from their medical history to have interstitial lung fibrosis or any other lung disease from our study population.

Tumor markers measurement

Serum KL-6 was measured by the sandwich enzyme immunoassay method using the KL-6 antibody (Ab) as both the capture and tracer Ab (14) according to the manufacturer's instructions (Eisai, Tokyo, Japan). KL-6 cut-off point was set at 500 U/L for this study. Assessment of alpha fetoprotein (AFP) and protein-induced vitamin K deficiency or absence (PIVKA-II) was performed using commercially available kits. Cut-off points were set at 10 ng/mL for AFP and 40 mAU/L for PIVKA-II.

Statistical analysis

Univariate statistical analysis was performed using Student's *t*-test for quantitative and χ^2 test with Yates' correction for qualitative data. Fisher's exact test was used

for comparison of small numbers; statistical significant level was set at $P<0.05$. Statistical analysis was performed using a computer software (SPSS, version 6.0).

RESULTS

Population background

A difference in mean age, prevalence of advanced Child class and HBV infection was observed between Egyptian and Japanese patients with HCC (Table 1). However, no difference in tumor characteristics was found between the two studied populations (Table 2).

KL-6 and other tumor markers in HCC

A significantly higher mean serum KL-6 (556±467) was found in HCC in comparison with non-HCC groups of patients with (391±176; $P<0.001$) and without (361±161; $P<0.001$) liver cirrhosis (LC). Serum KL-6 level did not correlate with either AFP (Figure 1) or PIVKA-II (Figure 1) serU/Levels. Using receiver operation characteristic (ROC) curve, the KL-6 level that gave the best sensitivity (61%) was found to be 334 U/L with a specificity of 50%, while PIVKA-II and AFP showed a sensitivity/specificity of (60/72)% and (80/78)% respectively. However, according to the manufacturer's instructions; a cut-off point of 500 U/L was used in this study that showed the highest specificity (80%) for KL-6 in comparison with the other two markers. Combining the values of KL-6; AFP and PIVKA-II resulted in improvement in the specificity of AFP for HCC diagnosis from 78% for AFP alone; 93% for AFP plus PIVKA-II to 99% for both plus KL-6 ($P<0.001$) (Table 3).

Table 2 Comparison of background tumor characteristics between Egyptian and Japanese HCC patients

Tumor characteristic	Egyptian (n = 65)	Japanese (n = 45)	P
Tumor multiplicity			
Solitary	25 (38)	22 (49)	>0.2
Multiple	40 (62)	23 (51)	0.06
Tumor size			
<3 cm	32 (49)	20 (44)	>0.2
3:< 5cm	15 (23)	14 (31)	>0.2
⩾5 cm	18 (28)	11 (25)	0.13
Metastases	01 (2)	>0.2	
Tumor grade[1]			
Well differentiated	5 (16)	2 (8)	>0.2
Poorly differentiated	5 (16)	4 (16)	>0.2

[1]Tumor grade is analyzed in 32 of the Egyptian and 22 of the Japanese groups who passed HCC resection operation during the study period.

Factors associated with positive KL-6 in the study population

Univariate analysis (Table 4) of possible factors that could be associated with elevated serum KL-6 in our study group showed that elevated AFP ($P<0.001$), Child's class C ($P = 0.002$), Egyptian race ($P = 0.003$) and HCC ($P = 0.008$) were significantly associated with positive serum KL-6. Also mean serum alkaline phosphatase level was significantly higher in KL-6 positive (564±475) in comparison with KL-6 negative (505±469) HCC patients ($P = 0.021$), but such a difference was not found among non-HCC corresponding group (Table 5). Mean serum bilirubin was found to be higher in KL-6 positive subgroups in both HCC and non-HCC ($P = 0.077$, 0.023) respectively, while mean serum albumin was significantly lower in both groups ($P = 0.029$, 0.041), respectively (Table 5).

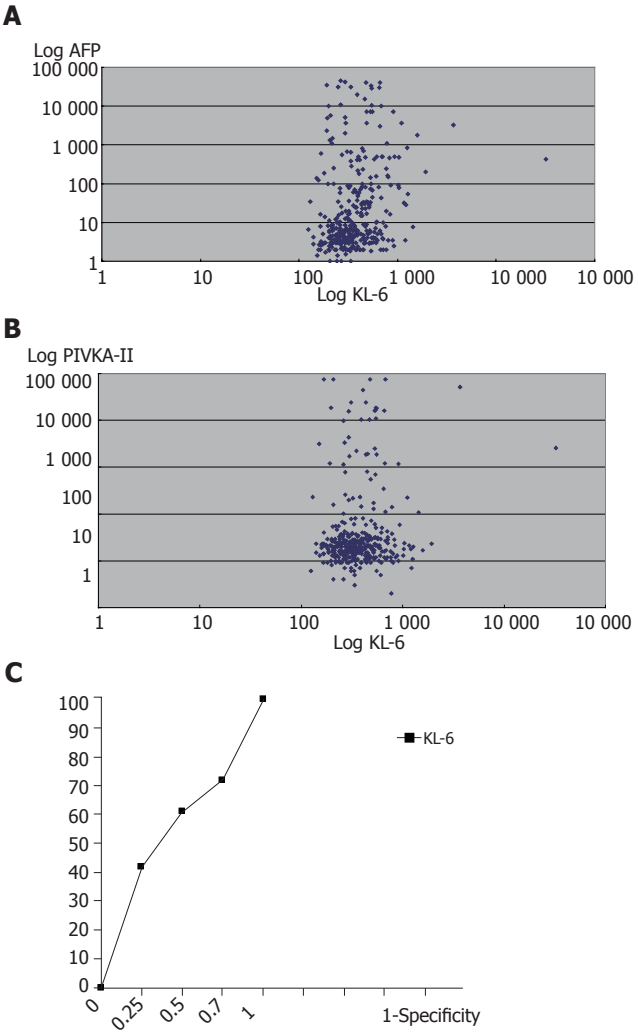

Figure 1 A: Correlation between KL-6 U/L and AFP ng/ml serU/Levels in the study population. $C = 0.04$, $P>0.1$. The Log values of both markers are shown; **B:** Correlation between KL-6 U/L and PIVKA-II mAU/L serU/Levels in the study population. $C = 0.03$, $P>0.5$. The Log values of both markers are shown; **C:** Receiver operating characteristic curves for KL-6 as predictors of HCC. The area under the ROC was found to be 0.574 (95%CI = 0.50–0.64). The best KL-6 sensitivity was obtained at a cut-off point = 334 U/L.

KL-6 in Egyptian vs Japanese

Mean KL-6 was significantly higher in Egyptians (576±522) in comparison with Japanese (510±300) HCC

Table 3 Comparison of the result of different tumor markers between HCC and non-HCC group

Tumor marker (cut-off point)	Sensitivity %	Specificity %
AFP (10 ng/mL)	86	78
PIVKA-II (35 mAU/L)	61	72
KL-6 (500 U/L)	34	80
AFP+PIVKA-II	86[1]	93
AFP+KL-6	87[2]	94
AFP+PIVKA-II+KL-6	87	99

[1]All PIVKA-II (+) HCC patients are AFP (+). [2]One KL-6 (+) HCC patient is AFP (–).

Table 4 Factors associated with KL-6 positivity in the study population

Factors	Total	KL-6 (+)	KL-6 (–)
Age (yr)			
>=50	244	60 (24)	184 (75)
<50	100	24(24)	76 (76)
P	NS		
Sex			
Male	257	61 (24)	196 (76)
Female	87	23 (26)	64 (73)
P	NS		
Ethnicity			
Egyptian	171	53 (31)	118 (69)
Japanese	173	31 (18)	142 (82)
P	0.003		
Underlying liver disease			
HCV-related	267	71 (27)	196 (73)
HBV-related	42	5 (12)	37 (88)
Non-viral	35	8 (23)	27 (77)
P	NS		
Cirrhosis			
(+)	169	46 (27)	123 (73)
(–)	175	38 (22)	137 (78)
P	NS		
Child's class			
C	47	20 (43)	27 (57)
A&B	297	64 (21)	233 (78)
P	0.002		
HCC:			
(+)	110	37 (34)	73 (66)
(–)	234	47 (20)	187 (80)
P	0.008		
AFP			
(+)	145	49 (34)	96 (66)
(–)	199	35 (17)	164 (82)
P	<0.001		

patients ($P = 0.041$) (Table 6). Although a significant difference in mean KL-6 level between HCC and non-HCC was observed in both Egyptian and Japanese patients with chronic liver disease ($P<0.001$ respectively), the difference was not statistically significant among Japanese patients with HCV-related disease (Table 6). No difference in mean KL-6 level was found between cirrhotic and non-cirrhotic in either HCC or non-HCC patients.

KL-6 and tumor characteristics

In the HCC groups of both Egyptian and Japanese patients; KL-6 showed no significant association with tumor site, echogenicity or multiplicity. However, a significantly lower mean KL-6 (Table 7) was noticed in larger size tumors of >5 cm (371±168 U/L) in comparison with tumors of less than or equal to 5 cm (537±323) ($P<0.05$ in the Japanese group).

DISCUSSION

KL-6 was studied as a tumor marker in different malignancies like breast, lung and pancreatic cancer and it was reported to be elevated in up to 50% of these malignancies[14]. Two previous studies by Moriyama *et al*.[19, 20] addressed KL-6 as a tumor marker for HCC in patients with HCV-related chronic liver disease, and his results showed that the estimated cumulative incidence of HCC development in HCV-related chronic liver disease patients was significantly greater in patients with positive KL-6[19] and suggested KL-6 to be used as a serological marker for HCC development in HCV-positive patients[20]. In our study, we included consecutive patients with chronic liver disease seen at outpatient settings in two different ethnic groups of possible different risk factors for HCC[22, 23] in order to have a wider spectrum of disease to judge KL-6 validity as a diagnostic test for HCC; however, one limitation was that most of the encountered patients in the two settings were actually with HCV-related disease with low proportion of HBV and non-viral-related disease. Our results showed a significantly higher mean KL-6 in HCC compared with non-HCC; either with or without LC; in addition no difference in mean KL-6 was found among HCC patients with and without LC; such findings together point to KL-6 association with HCC independent on the presence or absence of LC. A significantly higher mean KL-6 level was found in HBV-related in comparison with HCV-related HCC in both Egyptian and Japanese populations; a finding that deserves future study on a larger population of HBV-related disease. Our results also showed a significantly higher mean KL-6 level in HCC patients of Egyptian compared with Japanese race. The finding of a difference in the clinical background between both in terms of lower mean age and lower prevalence of HBV-related HCC could reflect a difference in the risk factors for HCC in both groups. Also, a higher prevalence of advanced Child class in the HCC Egyptian patients was observed that could stand behind the finding of higher mean KL-6 level in this group compared to their corresponding Japanese group. Although we excluded patients with overt schistosomal from this study, still some Egyptian patients had a past history of schistosomiasis with US evidence of hepatic periportal fibrosis (denoting a background of schistosomal liver disease) that could also explain the finding of higher mean KL-6 level in Egyptian HCC patients, if we consider the possibility that KL-6 could be a fibrosis marker too[21]. This topic is highly suggested for future study.

Table 5 Comparison of the clinical profile of KL-6 positive and negative patients with and without HCC

	HCC (+)			HCC (–)		
	KL-6 (+)	KL-6 (–)	*P*	KL-6 (+)	KL-6 (–)	*P*
	n = 37	*n* = 73		*n* = 47	*n* = 187	
Mean age (yr)[1]	59±12	62±10	NS	57±12	56±13	NS
Cirrhosis	39 (81)	55 (76)	NS	16 (34)	65 (35)	NS
Child's C	13 (35)	15 (21)	NS	7 (15)	11 (6)	0.045
Mean ALT	74±101	62±61	NS	59±33	57±45	NS
Serum						
Albumin (g/L)[1]	2.9±0.7	3.3±0.7	0.029	3.5±0.9	3.8±0.8	0.041
Bilirubin (mmol/L)[1]	2.7±2.8	2.5±3.0	0.077	2.4±2.9	1.4±1.9	0.023
ALP (IU/L)[1]	564±475	505±469	0.021	316±139	299±152	NS
AFP (+)	36 (97)	58 (80)	0.013	13 (28)	37 (20)	NS
PIVIKA (+)	23 (62)	44 (60)	NS	14 (30)	50 (27)	NS

[1]Data is shown as mean±SD. Other data is shown as *n* (%).

Table 6 Comparison of mean serum KL-6 level among different study sub-groups

	Egyptian			Japanese		
	HCC (+)	HCC (-)	*P*	HCC (+)	HCC (-)	*P*
Chronic liver disease[1]:	576 (522)	398 (185)	0.001	510 (300)	350 (147)	<0.001
HCV-related	558 (524)	400 (172)	0.008	356 (290)	382 (209)	>0.2
HBV-related	778 (663)	246 (72)	>0.2	877 (292)	340 (163)	<0.001
Non-viral	729 (538)	446 (309)	>0.2	262[2] ()	357 (160)	–
Cirrhotics	599 (586)	406 (159)	0.035	510 (350)	374 (196)	0.035
Non-cirrhotics	518 (325)	398 (185)	0.045	225 (73)	349 (222)	<0.001

[1]The KL-6 values are shown as mean (SD) U/L. [2]Only one patient's data.

Table 7 Difference in mean KL-6 level according to HCC size

	Egyptian	*P*[1] value	Japanese	*P* value
	(*n* = 65)		(*n* = 45)	
Tumor size				
<3 cm	485±227		618±361	
3:<5 cm	643±685	>0.1	456±285	0.17
⩾5 cm	581±420	>0.1	371±168	0.04

[1]*P* value is shown for the difference group (<3 cm) and the other two groups.

We used a cut-off point of 500 U/L for KL-6 positivity in this study; however, applying the ROC analysis showed that a cut-off point of 334 U/L would give the best sensitivity in our study population of 60% compared with only 32% for a cut-off (500 U/L); however, the best specificity was obtained using the later. Moriyama *et al*. used a cut-off point of 300 U/L in his analysis of KL-6 in HCV-related disease[19,20]. KL-6 serU/Level did not correlate with either serum AFP or PIVKA-II levels, which points to its behavior independently from either of them and this may justify its clinical significance as an independent tumor marker for HCC diagnosis when considered with both AFP and PIVKA-II. Our results also supported this finding as AFP specificity for HCC diagnosis improved from 78% for AFP alone and 93% of both AFP and PIVKA-II to 99% when combined with KL-6. Univariate analysis showed that low serum albumin, hyperbilirubinemia and elevated ALP were significantly associated with positive KL-6 in HCC patients, while KL-6 showed no association with LC in turn, and this denotes a possible association between positive KL-6 and deterioration of hepatic condition in HCC patients independent from their cirrhotic status; a finding that might point to KL-6 as a predictor of tumor aggression and/or local or systemic metastasizing potential. A follow-up study is needed to confirm its exact role in this regard.

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Modulation of gene expression in MHCC97 cells by interferon alpha

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Abstract

AIM: To elucidate the molecular mechanisms of the inhibitory effects of IFN-α on tumor growth and metastasis in MHCC97 xenografts.

METHODS: Three thousand international units per milliliter of IFN-α-treated and -untreated MHCC97 cells were enrolled for gene expression analysis using cDNA microarray. The mRNA levels of several differentially expressed genes in cDNA microarray were further identified by Northern blot and RT-PCR.

RESULTS: A total of 190 differentially expressed genes including 151 IFN-α-repressed and 39 -stimulated genes or expressed sequence tags from 8 464 known human genes were found to be regulated by IFN-α in MHCC97. With a few exceptions, mRNA levels of the selected genes in RT-PCR and Northern blot were in good agreement with those in cDNA microarray.

CONCLUSION: IFN-α might exert its complicated anti-tumor effects on MHCC97 xenografts by regulating the expression of functional genes involved in cell metabolism, proliferation, morphogenesis, angiogenesis, and signaling.

Key words: Interferon α; cDNA microarray; Gene expression profile; HCC

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INTRODUCTION

Human hepatocellular carcinoma (HCC) is one of the most prevalent malignancies in China. Patients with HCC often die of tumor metastasis and recurrence even after curative resection. Recently, a metastatic human HCC model in nude mice (LCI-D20) and a series of HCC cell lines (MHCC97, MHCC97-H, MHCC97-L) with different metastatic potentials derived from LCI-D20 have been established in our institute[1,2]. Using this model, IFN-α significantly inhibits tumor growth and metastasis of MHCC97 xenografts has been found[3-5]. However, the underlying molecular mechanisms are still unclear.

IFN-α is a multifunctional cytokine capable of interfering with viral infection, inhibiting cell proliferation, regulating cell differentiation, as well as modulating immune response[6-9]. It is well known that these pleiotropic effects of IFN-α are mediated primarily through the transcriptional regulation of many different functional genes. Thanks to the rapid progress in human genetic projects; many functional human genes and expressed sequence tags (ESTs) are identified and released, which make us possible to use cDNA microarray to survey IFN-α-modulated genes in MHCC97 cells. In this study, we identified 190 differentially expressed genes from 8 464 known human genes, which might mediate various biological functions of IFN-α. These data provide us useful clues for further studying the anti-tumor mechanisms of IFN-α and finding the IFN-α mimics for HCC therapy.

MATERIALS AND METHODS

Cell culture

MHCC97, a metastatic HCC cell line derived from LCI-D20 xenografts, was cultured in high glucose Dulbecco's modified Eagle's medium (Gibco-BRL, NY, USA) supplemented with 10% fetal calf serum (Hyclone, UT, USA), 100 U/mL penicillin and 100 μg/mL streptomycin in 20-cm^2 tissue culture flasks. Cells were grown at 37 °C in a humidified atmosphere of 50 mL/L CO_2 and passaged every 3 d.

cDNA microarray analysis

A total of 8 464 cDNAs of known human genes (United Gene Holding, Ltd, Shanghai) were amplified by polymerase chain reaction (PCR) using universal primers and spotted onto silylated slides (CEL Associates,

Houston, TX, USA) using a Cartesian PixSys 7500 motion control robot (Cartesian Tech, Irvine, CA, USA) fitted with ChipMaker micro-spotting technology (TeleChem, Sunnyvale, CA, USA). After being hydrated, dried, cross linked and washed, the microarray was ready for use. Total RNA was isolated from IFN-α-treated and untreated (3 000 IU/mL, 16 h) cells using TRIzol (Gibco-BRL). cDNA probes were prepared by reverse transcription and purified according to the methods described by Schena *et al*.[10]. Then equal amount of cDNA from IFN-α-untreated and treated MHCC97 cells was labeled with Cy3-dUTP and Cy5-dUTP, respectively. The mixed Cy3/Cy5 probes were purified and dissolved in 20 μL of hybridization solution (0.75 mol/L NaCl, 0.075 mol/L sodium citrate, 0.4% SDS, 50% formamide, 0.1% Ficoll, 0.1% polyvinylpyrrolidone and 0.1% BSA). Microarrays were pre-hybridized with 0.5 mg/mL salmon sperm DNA at 42 °C for 6 h. After being extensively washed, the denatured (95 °C, 5 min) fluorescent-labeled probe mixture was applied onto the pre-hybridized chips and further hybridized at 42 °C for 15-17 h under a cover glass. Subsequently, chips were sequentially washed for 10 min at 60 °C with 2×SSC+0.2% SDS, 0.1×SSC+0.2% SDS and 0.1×SSC solutions and dried at room temperature (1× SSC: 150 mmol/L NaCl, 15 mmol/L sodium citrate). Both Cy3 and Cy5 fluorescent signals of hybridized chips were scanned by ScanArray 4000 (GSI Lumonics, MA, USA) and analyzed using Genepix Pro 3.0 software (BioDiscovery Inc., CA, USA). To minimize artifacts arising from low expression, only genes whose Cy3 and Cy5 fluorescent intensities were both over 200 counts, or genes whose Cy3 or Cy5 fluorescent intensity was over 800 were selected for calculating the normalization cofactor (ln(Cy5/Cy3)). Genes were identified as differentially expressed, if the ratio of Cy5/(Cy3×normalization cofactor) (Cy5/Cy3*) was more than 2 or less than 0.5.

Reverse transcription and polymerase chain reaction

MHCC97 cells (106) cultured in 20-cm^2 flasks were treated with 3 000 IU/mL IFN-α (Roche, Shanghai) for 0 or 16 h, and total RNA was extracted (RNeasy Mini Kit, QIAGEN Inc., CA, USA). One microgram RNA was used to set-up reverse transcription reactions (Gibco-BRL, NY, USA). Nine differentially expressed genes identified by cDNA microarray were selected for analysis by semi-quantitative PCR. Appropriate primers were designed using Primer3 software (http://www-genome.wi.mit.edu). γ-Actin was used as an internal standard. PCR reaction conditions and primer sequences are summarized in Table 1.

Northern blot analysis

Total RNA of 3 000 IU/mL IFN-α-treated or untreated MHCC97 cells was isolated as described above. Thirty microgram was separated by 1% agarose formaldehyde gel electrophoresis and transferred to a nylon membrane (Millipore, MA, USA) in 10×SSC by capillary blotting. The membrane was hybridized with the appropriate cDNA probe prepared from the human library of cDNA clones (Biostar Genechip Inc., Shanghai) and labeled with [α-^{32}P]dCTP (Yahui Biomedical, Beijing) using random primer (Ambion Inc., Austin, TX, USA).

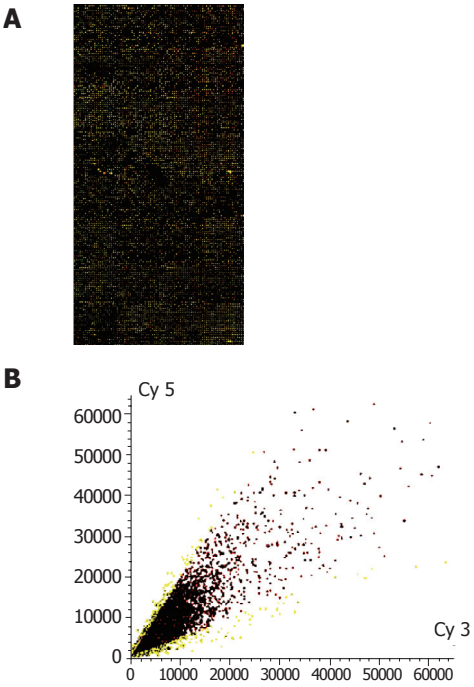

Figure 1 Representative hybrid result (**A**) and scatter plots (**B**) of cDNA microarray analysis in IFN-α treated MHCC97.

RESULTS

Gene expression profile identified by cDNA microarray

It is well known that the gene expression pattern of cells often varies with time and differentiation status and that cells derived from different individuals often have different genetic expression profiles. As a result, it is often difficult to extract useful information on the possible causes of phenotypic differences by comparing the genetic expression profiles of different cell lines. To minimize such complicated factors, we compared the gene expression profiles in 3 000 IU/mL IFN-α-treated and untreated (0 IU/mL) MHCC97 cells in two independent cDNA microarray analyses. We reasoned that such an internally consistent comparison might provide useful information on explaining the anti-tumor molecular mechanism of IFN-α in MHCC97 xenografts.

In 8 464 tested genes and ESTs, 190 genes were identified to be modulated by 3 000 IU/mL IFN-α treatment in MHCC97 cells. Among them the ex-pression of 151 genes was downregulated by IFN-α and the expression of 39 genes was upregulated by IFN-α. All differentially expressed genes are listed in Table 2 and the gene expression profiles obtained by cDNA microarray analysis are shown in Figure 1.

Nine differentially expressed genes evaluated by RT-PCR and Northern blot

To validate the results of cDNA microarray, we selected

Table 1 Primer sequence and condition for PCR analysis of selected genes

Category	Gene	Sense and antisense primers	Annealing (°C)	Cycles	Size (bp)
Cytoskeletal gene	Neutral calponin	5′-TGGCACCAGCTAGAAAACCT-3′; 5′-CAGGGACATGGAGGAGTTGT-3′	56	26	498
Proliferative gene	hMCM2	5′-ACCGAGACAATGACCTACGG-3′; 5′-CTAGCTGTCTGCCCCTTGTC-3′	56	30	382
Angiogenic gene	VEGF165 receptor	5′-GAAGCACCGAGAGAACAAGG-3; 5′-CACCTGTGAGCTGGAAGTCA-3′	56	30	359
IFN-α-induced genes	9-27	5′-TTGGTCCCTGGCTAATTCAC-3′; 5′-ATGAGGATGCCCAGAATCAG-3′	53	35	491
	ISG-56 ku	5′-AAAAGCCCACATTTGAGGTG-3′; 5′-GGCTGATATCTGGGTGCCTA-3′	54	30	451
MAPK pathway-related genes	ERK activator kinase (MEK2)	5′-CGAAAGGATCTCAGAGCTGG-3′; 5′-GTGCTTCTCTCGGAGGTACG-3′	56	26	349
	G3BP2	5′-GCAGAACCTGTTTCTCTGCC-3′; 5′-CACCACCACCTCTGGTTTCT-3′	56	30	475
	CHED	5′-TCCTTGGCGAACTCTTCACT-3′; 5′-TGCCATAAAGGGAGATCTGG-3′	56	30	336
cAMP/PI3 pathway-related gene	Adenylyl cyclase	5′-CCAGGAGCCTGAAGAATGAG-3′; 5′-GGCTTCTGAGCTCCAATCAC-3′	53	35	439
Housekeeping gene	γ-Actin	5′-ATGGAAGAAGAAATCGCCGC-3′; 5′-ACACGCAGCTCGTTGTAGAA-3′	55	25	287

nine genes whose expressions were clearly altered by IFN-α and evaluated their expressions by PCR and Northern blot. We enrolled IFN-α-regulated genes and found that the results were consistent with the previous reports[11,12].

For PCR analysis, we synthesized primers as indicated in Table 1 and performed semi-quantitative RT-PCR as outlined under "Materials and methods" after treatment of MHCC97 cells with 3 000 IU/mL IFN-α for 0 or 16 h. The transcription patterns of the same genes were also analyzed by Northern blot. Among the nine selected genes, seven downregulated genes were proved by cDNA microarray, six by RT-PCR and five by Northern blot analysis. Two stimulated genes, ISG-56 ku and 9-27 were proved by cDNA microarray, RT-PCR and Northern blot analysis. ERK activator kinase (MEK2), one repressed gene in cDNA microarray, was not changed in RT-PCR or Northern blot analysis. Thus, with a few exceptions, the results of RT-PCR and Northern blot were in good agreement with those of cDNA microarray analysis (Figure 2).

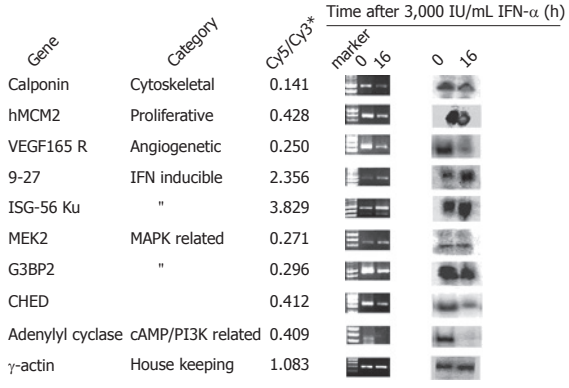

Figure 2 Confirmation of gene expression profiles in cDNA microarray analysis with RT-PCR and Northern blot.

DISCUSSION

cDNA microarray is a useful technique for rapid screening of gene expressions in cells, although the results need to be further confirmed by other molecular methods. Using this method, we found 211 hybrid dots, whose Cy5/Cy3* ratio was either more than 2 or less than 0.5 in IFN-α-treated MHCC97. Blasting the cDNA sequences in public database showed that these dots represented 190 different human genes or ESTs due to the redundant hybrids. Based on the results of RT-PCR and Northern blot, we believe that our cDNA microarray data are reliable. These differentially expressed genes might mediate the multiple biological functions of IFN-α directly or indirectly in MHCC97. We have artificially categorized these genes into nine functional clusters (Table 2).

IFN-α might interfere with cellular metabolisms by downregulating metabolic gene expression. In detail, IFN-α can inhibit glycolysis, glycogen degradation, gluconeogenesis as well as creatine or glucose transportation by repressing the expressions of liver-type phosphofructokinase (hPFKL), M2-type pyruvate kinase, brain glycogen phosphorylase, 2-oxoglutarate dehydrogenase, glucose transporter glycoprotein (SGLT) and cytosolic thyroid hormone-binding protein[13]. IFN-α can also inhibit lipolysis by reducing the expression of delta7-sterol reductase and pristanoyl-CoA oxidase, two key enzymes in lipid metabolism[14,15]. In addition, IFN-α reduces purine and pyridine biosynthesis by repressing the expression of GARs-AIRs-GART and serine hydroxymethyltransferase 2 (SHMT2). All these indicate that IFN-α-treated MHCC97 can result in lower ATP production and DNA synthesis, and slow down cell proliferation.

Many proliferation-, apoptosis- and cell cycle-regulating genes are modulated by IFN-α in MHCC97.

Table 2 Gene expression profile of MHCC97 cells induced by IFN-α

Category	GenBank ID	Gene description	Cy5/Cy3* (average)
2.1 Metabolism related genes	HUMCRTR	Creatine transporter	0.251
	HSGAGMR	GARs-AIRs-GART	0.289
	HUM2OGDH	2-Oxoglutarate dehydrogenase	0.298
	AF034544	Delta7-sterol reductase	0.318
	HUMTK	Thymidine kinase	0.333
	HSU12778	Acyl-CoA dehydrogenase	0.341
	HUMTHBP	Thyroid hormone-binding protein(p55)	0.349
	AF067127	7-Dehydrocholesterol reductase (DHCR)	0.356
	HSPRCOX	Pristanoyl-CoA oxidase	0.364
	AF035429	Cytochrome oxidase subunit 1	0.372
	AF070544	Glucose transporter glycoprotein (SGLT)	0.379
	HSPFKLA	Liver-type1-phosphofructokianse (PFKL)	0.392
	HUMSHMT	Serine hydroxymethyltransferase 2 (SHMT2)	0.407
	HUMMGPHB	Brain glycogen phosphorylase	0.413
	HUMTCBA	Cytosolic thyroid hormone-binding protein (p58)	0.415
	D88152	Acetyl-coenzyme A transporter	0.451
	HUMPKM2L	M2-type pyruvate kinase	0.456
	HSLDHBR	Lactate dehydrogenase B	2.156
	AF108211	Inorganic pyrophosphatase	2.25
	HSCOXVII	Cytochrome C oxidase VII	2.279
	HUMCYCPSK	Cytochrome C (HS7)	2.574
	HUMDBI	Diazepam binding inhibitor	2.628
2.2 Proliferation, apoptosis and damaged DNA repairing related genes	HSATPBR	Na/K ATPase beta subunit	0.208
	HUMP53T	Mutant p53 protein	0.233
	HSMITG	Mitochondrial DNA	0.309
	HSNUMAMR	Nuclear mitotic apparatus protein	0.325
	HSDNALIG3	DNA ligase III	0.34
	G28520	STS HSGC-31478 (homolog to Rad23a)	0.341
	AF096870	Estrogen-responsive B box protein	0.352
	AF001609	EXT like protein 3	0.367
	AF015283	Selenoprotein W	0.369
	AF011905	Putative checkpoint control protein hRad1	0.398
	HUMHMAM2	Minichromosome maintenance 2	0.408
	HUMRNAPII	RNA polymerase II 23 ku subunit	0.408
	AF007790	Inversely correlated with estrogen receptor Expression (ICERE-1)	0.413
	HSU78310	Pescadillo	0.43
	AF004162	Nickel-specific induction protein (Cap43)	0.434
	HSU3298	UV-damaged DNA binding factor	0.437
	HUMP1CDC47	P1cdc47	0.442
	HSU72649	B cell translocation gene 2	0.444
	AF031523	bcl-xL/bcl-2 associated death promoter (BAD)	0.481
	AF132973	CGI-39 (homolog to GRIM-19)	2.079
2.3 Morphogenesis, adhesion, and cytoskeleton remodeling related genes	D38735	Neutral calponin	0.141
	AF006082	Actin-related protein Arp2	0.197
	U01244	Fibulin 1D	0.212
	AF070593	Beta tublin	0.236
	HSU35622	EWS-E1A-F chimeric protein	0.255
	AF049259	Keratin 13	0.335
	HSPRO4HY	Prolyl 4-hydoxylase beta	0.337
	HUMCN4GEL	Collagenase type IV	0.36
	AF005654	Actin-binding double zinc-finger protein	0.378
	HSTEST	Testican	0.379
	HUMEPSURAN	Surface antigen	0.389
	AF004841	CAM-related/down-regulated by oncogenes	0.398
	HUMGLBA	Co-beta-glucosidase	0.402
	HUMCA1XIA	Alpha-1 type XI collagen	0.423
	HUMMCPGV	Macrophage capping protein	0.461
	HUMNID	Nidogen	0.497
	HSTUMP	Translationally controlled tumor protein	2.022
2.4 Signal transmitting related genes	HUMEPHT2R	Protein tyrosine kinase (NET PTK)	0.248
	HUMMEK2NF	ERK activator kinase (MEK2)	0.271
	HUMBADPTA	Beta-adaptin	0.273
	HUMP2A	Alpha-PR65	0.282
	HUMHRGAA	rab GDI alpha	0.285
	AF053535	ras-GAP/RNA binding protein G3BP2	0.296
	HSRING3GE	RING 3	0.316
	HSU45973	Pt Ins (4,5) P(2) 5-phosphatase	0.324
	HSU07139	Voltage-gated calcium channel beta	0.329
	HUMFTPB	Farnesyl-protein transferase beta	0.345
	HSU33053	Lipid-activated protein kinase (PRK1)	0.352
	HUMHK1A	Calcium-ATPase (HK1)	0.386
	HSU66406	EPH-related PTK receptor ligand LERK-8	0.386
	HSPP15	Placental protein 15	0.387
	HSADCYCL	Adenylyl cyclase	0.409
	HUMCHED	cdc2-related protein kinase (CHED)	0.412
	AF093265	Homer 3	0.415
	HSU40282	Integrin-linked kinase	0.416
	HUMGKAS	Stimulatory G protein	0.416
	HSU43939	Nuclear transport factor 2	0.429
	HUMCAK	Tyrosine protein kinase (CAK)	0.439
	HUMGNOS48	Endothelial nitric oxide synthase	0.443
	HUMCDPKIV	Calmodulin-dependent protein kinase IV	0.449
	HSPKX1MR	Protein kinase, PKX1	0.469
	D83760	Mother against dpp (Mad) related protein	0.472
	HUMEGFGRBA	EGF receptor binding protein GRB2	0.481
	HSU51004	Protein kinase C inhibitor (PKCI-1)	2.223
2.5 Tumor angiogenesis related genes	HUMRNAMBPE	Golli-mbp	0.236
	AF016050	VEGF 165 receptor/ neuropilin	0.25
	AF001307	Aryl hydrocarbon receptor nuclear translocator	0.27
	HSU64791	Golgi membrane sialoglycoprotein MG 160	0.355
	HUMPTPRZ	Protein tyrosine phosphatase Zeta-polypeptide	0.363
	HSU28811	Cysteine-rich FGFR (CFR1)	0.414
	HSU20758	Osteopontin	2.193

	HUMTR107	DNA-binding protein, TAXREB107	2.24
	HUMNEPPON	Nephropontin	2.413
2.6 Transcriptional activity related genes	S66431	Retinoblastoma binding protein 2	0.182
	HUMANT61K	Medium antigen-associated 61 ku protein	0.183
	HSU58197	Interleukin enhancer binging factor 2	0.226
	HSUBP	Upstream binding factor	0.266
	4758315	ets-related molecule, ETV5	0.267
	AF099013	Glucocorticoid modulatory element binding protein 1	0.309
	HSU72621	Lost on transformation 1(LOT1)	0.313
	HUMFOS	Oncogene protein, c-fos	0.361
	AB019524	Nuclear receptor co-repressor	0.369
	HS14AGGRE	Conserved gene telomeric to alpha globin cluster	0.398
	HSU74667	tat interactive protein (tip60)	0.404
	AF114816	KRAB-zinc finger protein SZF1-1	0.406
	HSU80456	Drosophila single-minded, SIM2	0.409
	AF117756	TRAP 150	0.41
	HSU15306	Cysteine rich DNA binding protein NFX1	0.417
	S57153	Retinoblastoma binding protein 1	0.469
	HUM56KDAPR	IEF SSP 9502	2.183
	HUMTR107	DNA binding protein. TAXREB 107	2.24
	HUMMSS1	Mammalian suppressor of sgv 1, MSS 1	2.313
2.7 mRNA and protein processing, secretory, proteolysis related genes	HSU39412	Alpha SNAP	0.141
	HSU47927	Isopeptidase T (ISOT)	0.229
	HSU72355	hsp27 ERE-TATA bind protein, HET	0.231
	AF077039	TIM17 homolog	0.238
	HUMHRH1	RNA helicase, HRH1	0.251
	AF206402	U5 SnRNP 100 ku protein	0.255
	D85429	Heat shock protein 40	0.344
	HSU85946	hSec 10p	0.378
	HSY10806	Arginine methyltransferase	0.412
	AB002135	Glycophosphatidylinositol anchor attachment 1	0.428
	AB007510	PRP8 protein	0.436
	HSU24105	Coatomer protein (COPA)	0.455
	HSCANPX	Calpain-like protease (CANPX)	0.456
	HSRBPRL7A	Ribosomal protein L7	2.067
	D89678	A+U-rich element RNA-binding protein	2.069
	HSU14966	Ribosomal protein L5	2.113
	HSRPL31	Ribosomal protein L31	2.142
	HUMPSC9	Proteasome subunit HC9	2.179
	HSU26312	Heterochromatin protein HP1 HS-gamma	2.182
	HUMRPS7A	Ribosomal protein S7	2.289
	AF106622	TIM17a	2.312
	HSUCEH3	Ubiquitin-conjugated enzyme UbCH2	2.323
	HUMRPS7A	Ribosomal protein S7	2.289
	AF106622	TIM17a	2.312
	HSUCEH3	Ubiquitin-conjugated enzyme UbCH2	2.323
	HUMRPS25	Ribosomal protein S25	2.326
	HUMRPSA3A	Ribosomal protein S3a	2.328
	HSRNASMG	Sm protein G	2.334
	HUMRPS18	Ribosomal protein S18	2.341
	HUMRP4SX	Ribosomal protein S4 isoform	2.346
	HUMPSC3	Proteasome subunit HC3	2.368
	HUMTCP20	Chaperonin protein, TCP20	2.572
	4504522	Chaperonin protein, hsp10	2.686
2.8 Tumor antigen processing, anti-viral infection related genes	HUMSAPC1	Cerebroside sulfate activator protein	0.211
	AF077011	Interleukin 16	0.23
	AF057307	Prosaposin	0.26
	HUMSIATA	Sialyltransferase	0.26
	AF055008	Epithelin 1 and 2	0.363
	HSU58766	FX protein	0.393
	HUMOSF1	OSF1	0.407
	HSU46194	RAGE 4	0.43
	HSU18121	136 ku double-stranded RNA binding protein	0.469
	AF021315	Reverse transcriptase	0.483
	S74095	Preproenkephalin A	2.115
	HUM927A	Interferon inducible protein 9-27	2.356
	HSIFI56R	Interferon inducible protein 56 ku	3.829
	HUMHCAMAP1	Interferon inducible protein 44 ku	4.03
2.9 Genes with unknown biological functions	D50928	KIAA0138	0.23
	AF132942	CGI08	0.269
	AB020677	KIAA0870	0.271
	AB011110	KIAA0538	0.277
	AB028956	KIAA1033	0.28
	HSU10362	GB36b glycoprotein	0.335
	4579277	A homolog of proteasome regulatory S2	0.352
	AB002356	KIAA0358	0.371
	4505130	A homolog of MCM3	0.371
	AB029020	KIAA1097	0.381
	HS130N43		0.383
	HSU66406	Eplg8	0.386
	HSNIPSNA1	NIPSNAP1 protein	0.391
	AB002378	KIAA0380	0.405
	HSU90907	Regulatory subunit of P55 PIK	0.407
	AB208959	KIAA1036	0.414
	AB020658	KIAA0851	0.416
	AF035282		0.416
	AF000136		0.419
	HUMORFFA	KIAA0120	0.424
	D13699	KIAA0019	0.43
	HUMORFB1	KIAA0123	0.432
	AF151830	CGI72	0.436
	AB007900	KIAA0440	0.437
	AB014595	KIAA0695	0.439
	HSM800064		0.439
	HUMORFA04	KIAA0115	0.457
	HSU79287		0.462
	AF007149		0.473
	AF007135		2.147
	AF151875	CGI117	2.184
	AF151857	CGI99	2.326
	HUMRSC508	KIAA0020	2.45

Downregulating the expression of mutant p53, mitochondrial DNA, nuclear mitotic apparatus protein (NuMA), and RNA polymerase II 23 ku subunit (polR2) might cause cell cycle arrest[16,17]. Downregulating the expression of DNA ligase III, hRad1, minichromosome maintenance 2 (hMCM2) as well as UV-damaged DNA binding factor might hinder damaged DNA repairing[18,19]. Stimulating retinoid-IFN-induced mortality 19 (GRIM-19) expression might promote IFN-α-induced apoptosis[20].

Several genes functionally related to cell morphogenesis,

adhesion, and cytoskeleton remodeling are also modulated by IFN-α in MHCC97. For example, decreasing the expression of calponin, actin-related protein 2 (Arp2), fibulin 1D, beta-tublin and epidermal surface antigen (ESA), *etc.*, might damage mitotic spindle formation and might interfere with actin-based cell motility, migration, adhesion and morphogenesis[21-24]. Reducing the expression of prolyl 4-hydroxylase beta, a key enzyme in collagen biosynthesis and type IV collagenase, a tumor-derived extracellular matrix metalloproteases might block tumor invasion and metastasis. Although most genes in this category were first identified as IFN-α regulating genes, their roles in mediating IFN-α functions need to be further studied.

In this study, we found that many genes functionally related to signal transmitting were affected by IFN-α in MHCC97. By repressing the expressions of discoidin domain receptor, integrin-linked kinase, EPH-related tyrosine kinase (EPT2) and MEK2, *etc.*, IFN-α might block cellular signaling initiated by tyrosine-kinase receptors[25,26]. By modulating the expressions of Rab GDI, Ras-related GTP-binding proteins and farnesyl-protein transferase and nuclear transport factor (NTF2) and G3BP2, a Ras-GAP/RNA binding protein, IFN-α might interfere with GTP/GDP exchange and nuclear import, thus influencing the recycles and activities of ras and its homologs[27-29]. By attenuating the expressions of adenylyl cyclase (AC) and phosphatidylinositol 4,5-bisphosphate 5-phosphatase (PtdIns (4,5)P(2)5- phospharase), a catalyzer of phosphatidylinositol 4,5-bisphosphate and PRK1, IFN-α might decrease inositol polyphosphate levels in cytosol and might inhibit the serine/threonine-kinase activities through cAMP/ PI3P signal pathway[30,31]. All these changes might exert inhibitory effects of IFN-α on MAPK and PI3K signaling. In addition, other signaling pathways such as Ca(2+), NO and TGFβ/hMAD-dependent signaling pathways are suppressed by IFN-α as well[32,33]. Plausibly Jak/STATs pathway, the most important IFN-α signaling pathway, is confirmed not to be regulated in IFN-α-treated MHCC97. The deficient expression of p48 (ISGF3γ) in this cell line may be the possible mechanism for the non-response of IFN-α priming via Jak/STATs pathway (data not shown).

In this study, we found that many angiogenic-related genes were regulated by IFN-α. By attenuating the expressions of Golli-MBP[34], VEGF 165 receptor and aryl hydrocarbon receptor nuclear translocator (ARNT)[35] as well as Golgi membrane sialoglycoprotein MG 160, a bFGF binding protein and cysteine-rich FGF receptor (CFR-1)[36], IFN-α may destroy the balance between pro- and anti-angiogenic factors and exert its inhibitory effects on tumor angiogenesis.

It is well known that cells usually respond to various stimuli by rapidly shifting the functions of transcriptional factors. Using this strategy, IFN-α might impose its anti-proliferative functions and hormone response by fluctuating the expression of several transcriptional factors or their cofactors such as retinoblastoma binding protein2 (RBP2), interleukin enhancer binding factor 2, lost on transformation 1 (LOT1) and KRAB-zinc finger protein (SZF1)[37-40].

In addition, IFN-α might hinder with mRNA/rRNA spicing and maturation by downregulating RNA helicase (HRH1), U5 snRNP[41] and affect protein transportation, secretion and proteolysis by downregulating alpha SNAP, GPAA1, hSec10p, hsp40 and isopeptidase T, a putative molecular in ubiquitin–proteasome pathway[42-44]. Meanwhile IFN-α might evoke anti-viral or tumor immune response by upregulating 9-27, 56 ku protein and p44 expressions.

Except for functionally definite genes, many ESTs with unknown functions were identified as IFN-α-regulated genes in our study (Table 2). In conclusion, cDNA microarray is a useful, rapid method for screening transcriptome of cells and potentially paves a way for elucidating IFN-α effects on tumor growth and metastasis.

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Detection of germline mutations of *hMLH1* and *hMSH2* based on cDNA sequencing in China

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Abstract

AIM: To detect the germline mutations of *hMLH1* and *hMSH2* based on mRNA sequencing to identify hereditary non-polyposis colorectal cancer (HNPCC) families.

METHODS: Total RNA was extracted from peripheral blood of 14 members from 12 different families fulfilling Amsterdam criteria II. mRNA of *hMLH1* and *hMSH2* was reversed with special primers and heat-resistant reverse transcriptase. cDNA was amplified with expand long template PCR and cDNA sequencing analysis was followed.

RESULT: Seven germline mutations were found in 6 families (6/12, 50%), in 4 *hMLH1* and 3 *hMSH2* mutations (4/12, 33.3%); (3/12, 25%). The mutation types involved 4 missense, 1 silent and 1 frame shift mutations as well as 1 mutation in the non-coding area. Four out of the seven mutations have not been reported previously. The 4 *hMLH1* mutations were distributed in exons 8, 12, 16, and 19. The 3 *hMSH2* mutations were distributed in exons 1 and 2. Six out of the 7 mutations were pathological, which were distributed in 5 HNPCC families.

CONCLUSION: Germline mutations of *hMLH1* and *hMSH2* can be found based on cDNA sequencing so as to identify HNPCC family, which is highly sensitive and has the advantages of cost and time saving.

Key words: *hMLH1*; *hMSH2*; Colorectal cancer; Hereditary non-polyposis; Reverse transcription; Germline mutation

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INTRODUCTION

hMLH1 and *hMSH2* are the two most important genes for HNPCC, which is the most common hereditary colon syndrome accounting for 10% of all colorectal cancers. It is autosomally dominant with a penetrance rate of 80-90%. HNPCC occurrence is closely associated with deficiency or loss of function of mismatch repair (MMR) genes. Affected individuals have an approximately 70% lifetime risk of colon cancer with a mean onset age of 44 years and an approximately 40% lifetime risk of endometrial cancer in females. At least 5 MMR genes, *hMLH1, hMSH2, hMSH6, hPMS1,* and *hPMS2*, have been implicated in HNPCC[1,2]. Information of genetic linkage analysis shows that germline mutations of *hMLH1* and *hMSH2* account for nearly 90% of all germline mutations found in HNPCC[3]. Germline mutations in MMR genes predispose to colorectal and other HNPCC associated epithelial cancers. Identification of MMR gene germline mutations has direct clinical implications in counseling and management of HNPCC.

Methods such as microsatellite instability (MSI), immunohistochemistry (IHC)[4-6], and sequencing of genes are employed to screen HNPCC. The most specific method is to detect the germline mutations of MMR. Its cost and sensitivity limitations can be overcome at least in part by RNA-based analysis[7]. It is the first time in China that we identified HNPCC families by detecting germline mutations of *hMLH1* and *hMSH2* genes based on cDNA sequencing with special primers and heat-resistant reverse transcriptase.

MATERIALS AND METHODS

Subjects

Fourteen anticipants from 12 unrelated families fulfilling Amsterdam criteria II for HNPCC were studied. Personal and family cancer history was obtained from the patients and their relatives. Pathological diagnosis and death were confirmed by review of medical records, pathological reports or death certificates.

Samples

Three microliters of peripheral blood was taken from each participant. Total RNA was extracted using TRIzol (Sigma Company) according to the manufacturer's instructions.

RT-PCR

cDNA was synthesized with transcriptor reverse transcriptase (Roche Diagnostics) using 0.5 μg of total RNA and specific primers complementary to the 3' end of *hMLH1* (2484-TATGTTAAGACACATCTATTTATTTA-2459) and to the 3' end of *hMSH2* (3145-CCACCAAACTACA TGATTTTATTTATAAAATTC-3114). RT was performed at 60 °C for 60 min.

cDNA of *hMLH1* and *hMSH2* was amplified in two overlapping fragments using primers (Table 1) to generate products of ~2 000 bp. PCR was performed using expand long template PCR (Roche Diagnostics) at 94 °C for 5 min; then 10 cycles at 94 °C for 30 s, at 59 °C for 30 s, at 68 °C for 3 min; 32 cycles at 94 °C for 30 s, at 57 °C for 30 s, at 68 °C for 3 min with a final elongation at 68 °C for 7 min.

PCR products were size fractionated by agarose gel electrophoresis and analyzed by ethidium bromide staining.

Sequencing

Purified PCR fragments were sequenced directly using a DNA sequencing kit according to Applied Biosystems from USA with BigDye Terminators on an ABI3700 automated DNA sequencer.

cDNA of *hMLH1* (2 484 bp) was sequenced in six overlapping fragments and cDNA of *hMSH2* (3 145 bp) was sequenced in eight overlapping fragments using primers (Table 2).

Table 1 Sequence and localization of primers used for amplification of cDNA of *hMLH1* and *hMSH2*

Sense	Antisense
hMLH1-1F(1-18)	*hMLH1*-5R(2198-2175)
CTTGGCTCTTCTGGCGCC	GAGCGCAAGGCTTTATAGACAATG
hMLH1-4F(1333-1353)	*hMLH1*-6R(2484-2459)
GCTGAAGTGGCTGCCAAAAAT	TATGTTAAGACACATCTATTTATTTA
hMSH2-1F(1-21)	*hMSH2*-7R(2753-2732)
GGCGGGAAACAGCTTAGTGGG	GGGCATTTGTTTCACCTTGGAC
hMSH2-6F(1898-1920)	*hMSH2*-8R(3145-3114)
CGTGTCAAATGGAGCACCTGTTC	CCACAAACTACATGATTTTATTTATAAAATTC

RESULTS

The sizes of amplified *hMLH1* and *hMSH2* segments were respected (Figure 1). Seven germline mutations were found in 6 out of 12 families, 4 *hMLH1* and 3 *hMSH2* mutations (4/12, 33.3%); (3/12, 25%). The mutation types involved 4 missense, 1 silent and 1 frame shift mutations as well as 1 mutation in non-coding area, including *hMLH1* mutation in family H2 at 649 codon 217 exon 8: CGC→TGC; *hMLH1* mutation in family H31 at 1742 codon 581 exon 16: CCG→CTG; *hMLH1* missense mutation in family H114 at 1151 codon 384 exon 12: GTT→GAT; family H111 *hMLH1* non-coding area at 2438 exon 19: A→C; family H11 *hMSH2* at 14 codon 5: CCG→CAG; family H38 hMSH2 mutations at 295 and 296 codon 99 exon 2: 295: A→C, 296:del.G (Table 3, Figure 2).

DISCUSSION

Colorectal cancer (CRC) is one of the most common malignant tumors and its incidence is increasing gradually. According to the different molecular mechanism, CRC is divided into sporadic and genetic types. The latter type HNPCC is characterized by its early onset[8-10], location in the proximal colon and an increased risk of neoplasms in extracolonic organs including endometrium, stomach, urothelium, small intestine, ovary and multiple

Table 2 *hMLH1* and *hMSH2* primers used for sequencing of cDNA

Sense		Antisense	
hMLH1-1F	CCTGGCTCTTCTGGCGCC	*hMLH1*-1R	CTTTTCTCCTCGTGGCTATGTTGT
hMLH1-2F	ATGTGCTGGCAATCAAGGGA	*hMLH1*-2R	GGTGCACATTAACATCCACATTCT
hMLH1-3F	CCAAAAACACACACCCATTCCT	*hMLH1*-3R	CCTTTGTTGTATCCCCCTCCA
hMLH1-4F	GCTGAAGTGGCTGCCAAAAAT	*hMLH1*-4R	CATCTTCCTCTGTCCAGCCACTC
hMLH1-5F	TTGCCATGCTTGCCTTAGATAGTC	*hMLH1*-5R	GAGCGCAAGGCTTTATAGACAATG
hMLH1-6F	GCTCCATTCCAAACTCCT	*hMLH1*-6R	TATGTTAAGACACATCTATTTATTTA
hMSH2-1F	GGCGGGAAACAGCTTAGTGGG	*hMSH2*-1R	CTCTGGCCATCAACTGCGGAC
hMSH2-2F	GGCTTCTCCTGGCAATCTCTCTCA	*hMSH2*-2R	CTTGATTACCGCAGACAGTGATGAAAC
hMSH2-3F	GCAAAAAGGGAGAGCAGATGAATAGTG	*hMSH2*-3R	GGCAAGTCGGTTAAGATCTGGGAAT
hMSH2-4F	AGATGCAGAATTGAGGCAGACTTTACA	*hMSH2*-4R	GGACTTTTTCTTCCTTACAGGTTACACG
hMSH2-5F	CAGAGATCTTGGCTTGGACCCT	*hMSH2*-5R	TTCAACACAAGCATGCCTGGAT
hMSH2-6F	CGTGTCAAATGGAGCACCTGTTC	*hMSH2*-6R	GATTGGCCAAGGCAGTAAGTTCAT
hMSH2-7F	AATCATAGATGAATTGGGAAGAGGAACT	*hMSH2*-7R	GGGCATTTGTTTCACCTTGGAC
hMSH2-8F	CTATCTGGAAAGAGAGCAAGGTGAA	*hMSH2*-8R	CCACAAACTACATGATTTTATTTATAAAATTC

Table 3 *hMLH1* and *hMSH2* mutations detected by cDNA sequencing

Families	Genes	Exon	Codons affected	DNA change	Amino acid change	Mutation types
H31	*hMLH1*	16	581	T>C, at 1742	Pro→Leu	Missense
H111	*hMLH1*	19	Non-coding area	A>T, at 2438		
H114	*hMLH1*	12	384	T→A, at 1151	Val→Asp	Missense
H2	*hMLH1*	8	217	T->C, at 649	Arg→Cyc	Missense
H11	*hMLH1*	1	5	A>C, at 14	Pro→His	Missense
H38	*hMLH1*	2	99	A>C, at 295	Arg→Arg	Silent
H38	*hMLH1*	2	99	Del G, at 296	Frame shift	Frame shift

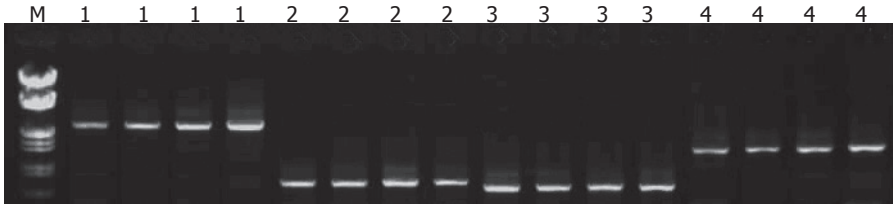

Figure 1 Amplified segments of *hMLH1* and *hMSH2*. "M" is mark. Lanes 1-4 sizes of segments.

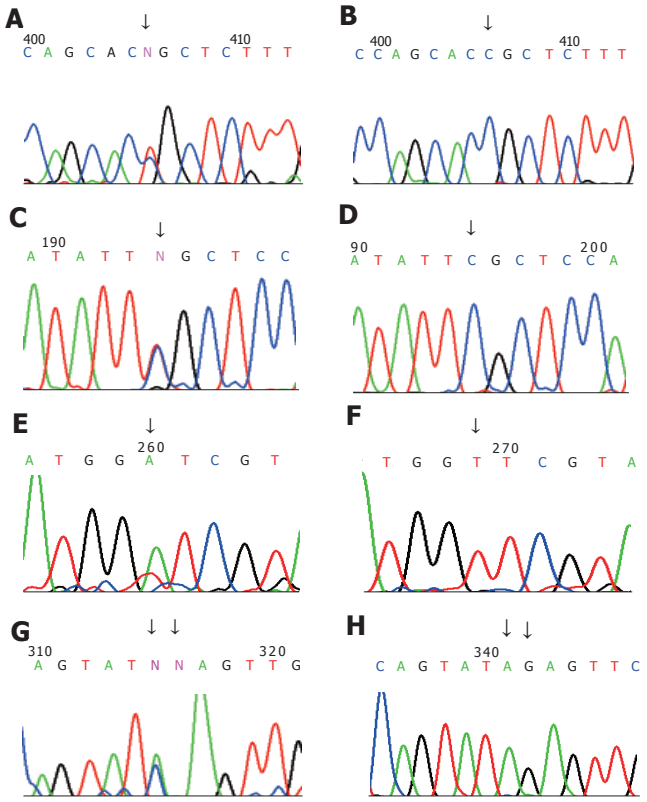

Figure 2 *hMLH1* and *hMSH2* mutations (**A, C, E, G**) and wild-type sequence (**B, D, F, H**) in families H31, H2, H114, and H38 at different codons. Arrows indicate the corresponding sites of mutation.

metachronous CRCs[9,11-14]. Its prognosis is better than sporadic type of CRC[15]. HNPCC is closely associated with the deficiency or loss of MMR gene function. Identification of MMR gene germline mutations has direct clinical implications in counseling and management of HNPCC.

Methods are available for the identification of HNPCC. The most specific method is to detect the germline mutations of MMR genes. Up to now, the germline mutations are mainly detected by genomic DNA-based sequencing (gDNA). A lot of information shows that the germline mutations of MMR genes associated with HNPCC are mainly localized in exons[3,5,7,13]. The gDNA-based sequencing is invariably affected by introns. cDNA-based sequencing of MMR genes has been reported recently. The new technique utilizes specific primers and heat-resistant reverse trancriptase to specifically synthesize cDNA of MMR genes, then full-length cDNA is amplified in two over fragments using specific primers followed by sequencing analysis of cDNA. The technique can successfully avoid the influence of introns. Additionally, it is well known that RNA is easily decayed. If RNA samples are stored too long, reverse transcription with random primers and common reverse transcription enzyme often fails, while the new technique employs specific primers and heat-resistant reverse trancriptase, the limitations can be overcome at least in part, thus improving the specificity and efficiency. Anna *et al*.[7] compared the two techniques and found that cDNA-based sequencing not only has the advantage of specificity and efficiency, but also a lower cost, being 2.5-3 times less expensive than gDNA-based sequencing. We used 35 pair primers to amplify the two genes in the past, and only 4 pair primers were used in the present study, the procedure is greatly simplified.

We detected 7 germline mutations in 14 anticipants with HNPCC from 12 different families employing the new technique. The 3 mutations, at sites 1151, 14, and 217 in *hMLH1* reported, the first two have been verified to be pathological. Moreover, the mutation at 1151 in *hMLH1* has been found only in Japan and Korea, which is likely to be a hot mutation site in East Asia. The mutation at site 217 in *hMLH1* occurs at a less conserved region is in 80 healthy Japanese. Whether it is pathological or not needs further study. None of the 4 unreported mutations belongs to polymorphism[17]. The 6 pathological mutations (2 reported, 4 unreported) were distributed in 5 HNPCC families in our study.

Of course, all mutations cannot be detected by the improved technique. For example, mutations in the promoter and 3'-untranslated regions of *hMLH1* and *hMSH2* cannot be detected. Sequencing of individual exons of gDNA also has such limitations.

Up to now, there is no optimal method to screen HNPCC patients or their families. The new technique can be utilized to screen HNPCC patients and their families, which may achieve a better result.

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Cytokine profile in Egyptian hepatitis C virus genotype-4 in relation to liver disease progression

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Abstract

AIM: To observe the imbalance between T helper cell Th1 and Th2 cytokines in several chronic hepatitis disease at different stages of disease progression.

METHODS: We measured the cytokine levels of Th1 (IL-2 and IL-2R), Th2 (IL-10) and the pro-inflammatory cytokines (IL-6 and IL-6R and TNF and TNF-RI and II) by the ELISA technique in the sera of 33 hepatocellular carcinoma (HCC) patients and 20 chronic liver disease (CLD) patients. In addition, 20 asymptomatic hepatitis C virus carriers and 20 healthy subjects negative for hepatitis C virus(HCV) markers served as controls.

RESULTS: Anti-HCV antibodies were found to be positive in 94% of HCC cases and 75% of CLD cases. On the other hand, HCV viremia was detected using RT-PCR in 67% of HCC cases and 65% of CLD cases. HBsAg was positive in 9% of HCC cases and 30% of CLD cases. Also bilharzial-Ab was positive in 55% of HCC cases, 65% of CLD cases and in 70% of asymptomatic carriers (ASC). HCC patients had significantly higher values of IL-2R, TNF-RII (P<0.001), and TNF-RI (P>0.05), but lower TNFα (P<0.001) and IL-6 (P = 0.032) in comparison to ASC. But, in comparison to non-cancer controls, HCC patients had higher values of IL-2R, IL-6R, TNF-RI and TNF-RII, but lower TNF-α (P<0.001). CLD patients had higher IL-2R, TNF-RI, and TNF-RII (P<0.001) than ASC. But, in comparison to non-cancer controls, CLD patients had higher values of IL-2R, TNF-RI and TNF-RII, but lower TNF-α (P<0.001). IL-10 was higher (though not significantly) in HCC and CLD patients than in symptomatic carriers and non-cancer controls.

CONCLUSION: Liver disease progression from CLD to HCC due to HCV genotype-4 infection is associated with an imbalance between Th1 and Th2 cytokines. IL-2R, TNF-RI, and TNF-RII could be used as potential markers.

Key words: Cytokine; HCV; Genotype-4; Liver disease

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INTRODUCTION

Hepatitis C virus(HCV) is a common cause of hepatocellular injury that is associated with complex and vigorous immunologic mechanisms. Both humoral and cell-mediated immune responses participate in the host defense against HCV infection, but it is increasingly recognized that cellmediated response to the cytokine system plays a role in the im-munopathogenesis of chronic hepatitis C[1].

Cytokines constitute a complex network of molecules involved in the regulation of the inflammatory response and the homeostasis of organ functions. Moreover, cytokines coordinate physiologic and pathologic processes going on in the liver, such as liver growth and regeneration, inflammatory processes including viral liver disease, liver fibrosis and cirrhosis. Liver growth and regeneration are regulated by several cytokines. The cell-mediated immune response plays a central role in hepatocellular necrosis and in the immunopathogenic mechanisms involved in viral clearance and persistence in liver disease of viral etiology[2].

T lymphocytes and immunoregulatory cytokines are of critical importance in the host defense against HCV infection. T-helper type 1 (Th1) cytokines (interleukin-2 [IL-2], interferon-γ [IFN-γ]) are required for host anti-viral responses, while T-helper type 2 (Th2) cytokines (IL-4, IL-10) can inhibit the development of these effectors[3]. It has been demonstrated that pro-inflammatory IL-6 is able

to influence hepatocarcinoma progression in patients with liver cancer[4]. Tumor necrosis factor (TNF) plays a role in the pathogenesis of chronic hepatitis C[5]. Also, chronic HCV infection is associated with an increase in the levels of soluble TNF receptors I and II[6].

Screening high-risk populations with ultrasonography and serum αFP levels produces diagnosis of only 40-60% of patients with hepatocellular carcinoma (HCC) at a stage where the tumor can be resected or treated with curative intent[7].

In this study, we aimed to characterize serum cytokine levels of IL-2 and its receptor (IL-2R), IL-6 and its receptor (IL-6R), IL-10, TNF-α and its soluble receptors (TNF-RI and TNF-RII) by enzyme immunosorbent assay in HCC patients, chronic liver disease (CLD) patients, and HCV asymptomatic carriers (ASC), to figure out the possible imbalance between Th1 and Th2-like cytokines and their possible relation to hepatocarcinogenesis, as well as to evaluate the clinical significance of these cytokines in different stages of HCV infection, and their possible use as markers of disease progression, since there are no reliable markers for disease progression from CLD to HCC.

PATIENTS AND METHODS

Patients and controls

This study was conducted on 33 histologically proven HCC and 20 CLD patients (i.e., chronic active hepatitis with or without cirrhosis). All patients were presented before treatment to the specialized liver clinic of the National Cancer Institute (NCI), Cairo University, between April 2000 and June 2001. The study also included 40 control subjects: 20 asymptomatic carriers of HCV infection (ASC) as positive controls (positive for both HCV-AB and HCV RT-PCR), and 20 subjects without infection with HCV (NC) as negative controls (negative for HCV by both anti-HCV-Ab and HCV RT-PCR). The criteria for inclusion in the study groups were as follows: (a) ASC group: persistently normal alanine aminotransferase (ALT) values for 6 mo and no detectable liver changes by sonography except for a bright fatty liver, which is common in the Egyptian population. (b) CLD group: (1) persistent increase of the ALT values more than three times the normal value for atleast 6 mo; (2) exclusion of other causes of CLD such as alcoholism or hepatotoxic drugs; (3) histopathological examination of core needle biopsies. Accordingly, patients were classified into 8 mild, 7 moderate, and 5 severe cases of CLD. (c) HCC group: HCC neoplastic cells were identified histopathologically in H&E-stained sections of a core needle biopsy. Cases were classified into G1 (8 cases), G2 (22 cases) and G3 (3 cases). A detailed history and physical examination of the patients were carried out with special emphasis on history of bilharzias, prior parenteral therapy, infective hepatitis and jaundice or other signs of liver cell failure. Complete clinical examination, which includes the manifestations of hepatitis and liver cell failure such as jaundice, hepatomegaly, tenderness in the right hypochondrium, ascites, spleenomegaly, lower limb edema as well as abdominal ultrasonography was also done side by side with routine laboratory investigations including complete blood picture, liver and kidney function tests.

ELISA and HA assays

Sera collected from 5 mL of coagulated blood was aliquoted and stored at -80 ℃ until use. All the sera of patients and controls were tested for HCV antibody and HBsAg by the third-generation ELISA using kits from Innogenetics (Belgium) and the Equipar (Saronno, Italy). They were also tested for antibodies of Schistosomal infestation by quantitative indirect hemagglutination kits from Fumouze Laboratories (Paris, France). All tests were done according to the manufacturer's instructions.

RT-PCR of HCV

Nucleic acid extraction was done by QIAGEN viral RNA Mini-extraction kit (QIAGEN) using 140 μL of patient serum according to the manufacturer's procedure.

RT and PCR were done as previously described by Zekri *et al.*[8]. After completion of the amplification reaction, 10 μL of each PCR reaction product was analyzed by electrophoresis through an agarose 1.2% gel stained by ethidium bromide in Tris-acetate-EDTA buffer (pH 8.0) and DNA was transferred from the gel onto a nitrocellulose filter with alkaline buffer (4 N NaOH). The transferred DNA was cross-linked by incubation for 2-3 h at 80 ℃ and the blot was then hybridized with an internal probe[8].

HCV genotyping

The line immuno-probe assay was used to determine the HCV genotype as described previously[9] using INNO-LiPA II and III provided by Innogenetics (Belgium).

ELISA for cytokine assay

The following cytokines were assayed for all study groups using quantitative ELISA plate method: IL-2 (Quantikine, R&D Systems, Inc., Minneapolis, USA), soluble IL-2 receptor (sIL-2R) (Diaclone Research, France), IL-10 (Quantikine R&D Systems, Inc., Minneapolis, USA), IL-6 (Accucyte, Cytimmune Sciences Inc., MD, USA), soluble IL-6 receptor (sIL-6R) (Diaclone Research, France), tumor necrosis factor alpha (TNF-α) (Accucyte, Cytimmune Sciences Inc., MD, USA), as well as their soluble receptors (sTNF-RI, sTNF-RII; Immunotech, France). We considered the cut-off values for the studied cytokines as mean+2SD of the negative controls.

Histological studies of liver

Liver core needle biopsies (atleast 10 mm long) from CAH and HCC patients who participated in the study were examined by two independent pathologists. Biopsy specimens were assessed for fibrosis (score 0-4) and activity (score 0-18) according to the scoring system of

Knodell. Chronic hepatitis C was defined as mild, if the total score was 6, moderate, if the score was between 6 and 9, and severe, if the score was 9.

Statistical analysis

SPSS package (version 10) was used. Mean and standard deviation were estimates of quantitative data. Non-parametric *t* test (Mann-Whitney test) or non-parametric ANOVA (Kruskal-Wallis test) was used to compare means of more than two independent groups. Fisher's exact and chi-square tests were used to validate the hypothesis of proportional independency. Correlation analysis was used to detect the association between quantitative data.

RESULTS

The clinical characteristics of the studied groups are shown in Table 1. HCC patients had significantly higher cirrhosis, irregular surface of liver, jaundice, serum AST and HCV-Ab positivity than in CLD cases (0.05, 0.007, 0.002, 0.006, and 0.05 respectively) and only HCC patients had significantly lower HBsAg than CLD patients. All the studied cases showed HCV genotype-4 by INNO-LiPA.

No significant difference was found in the history of bilharziasis, prior to the parenteral anti-bilharzial therapy, smoking, diabetes and hypertension in relation to the presence of HCV-Ab was found in HCC and CLD patients. HCV viremia by RT-PCR was positive in 22 of 31 (71%) HCV-Ab positive HCC cases, and in 11 of 15 (73%) HCV-Ab positive CLD cases.

Scatter diagram representing the values of IL-2, IL-2R, IL-6, IL-6R, IL-10, TNF-α and their soluble receptors TNF-RI and TNF-RII in HCC, CLD cases, ASC and normal controls around the cut-off value are shown in Figures 1A-G.

Concentrations of IL-2R, IL-6R and IL-10 were higher in HCC patients than in other groups (16.7±5.4 ng/mL, 56.5±17.97 ng/mL, 9±26.07 pg/mL respectively). On the other hand, the mean concentrations of TNF-RI and TNF-RII were higher (1.87±1.5 and 9.160±0.4 ng/mL) in CLD patients than in other groups. Schistosomal-Ab and TNF-α were higher (1205±120 and 811±5.8 ng/mL) in asymptomatic HCV carriers than in other groups. IL-6R was significantly higher in HCC, CLD and ASC than NC group and there is a consistent increase in the IL-10 level with the disease progression from NC to HCC (Table 2).

In HCC group, positive HCV-RT-PCR cases had higher values of IL-2R, IL-6, IL-10 and TNF-RII (P = 0.689, P = 0.925, P = 0.636 and P = 0.05 respectively) than non-viremic cases, whereas positive HCV-RT-PCR in CLD cases had higher values of IL-6R, IL-10 and TNF-α (P = 0.28, P = 0.08 and P = 0.966) (results not shown).

No significant difference was noticed in any of the clinical characteristics of the patients with cytokine values above the cut-off, when compared to those below the cut-off in both HCC and CLD patients (results not shown). Scatter diagram representing the values of IL-1, IL-2R, IL-6, IL-6R, IL-10, TNF-RI and TNF-RII in HCC, CLD cases, ASC and non-cancer controls (NC) around the cut-off value are shown in Figures 1A-G. Mean serum cytokine levels in the different study groups are shown in Table 2. Regarding IL-2, no difference in its level was observed among the four groups. However, HCC patients had significantly higher values of IL-2R, TNFRII (P<0.001), and TNF RI (P>0.05), but lower TNF-α (P<0.001) and IL-6 (P = 0.032) in comparison to ASC.

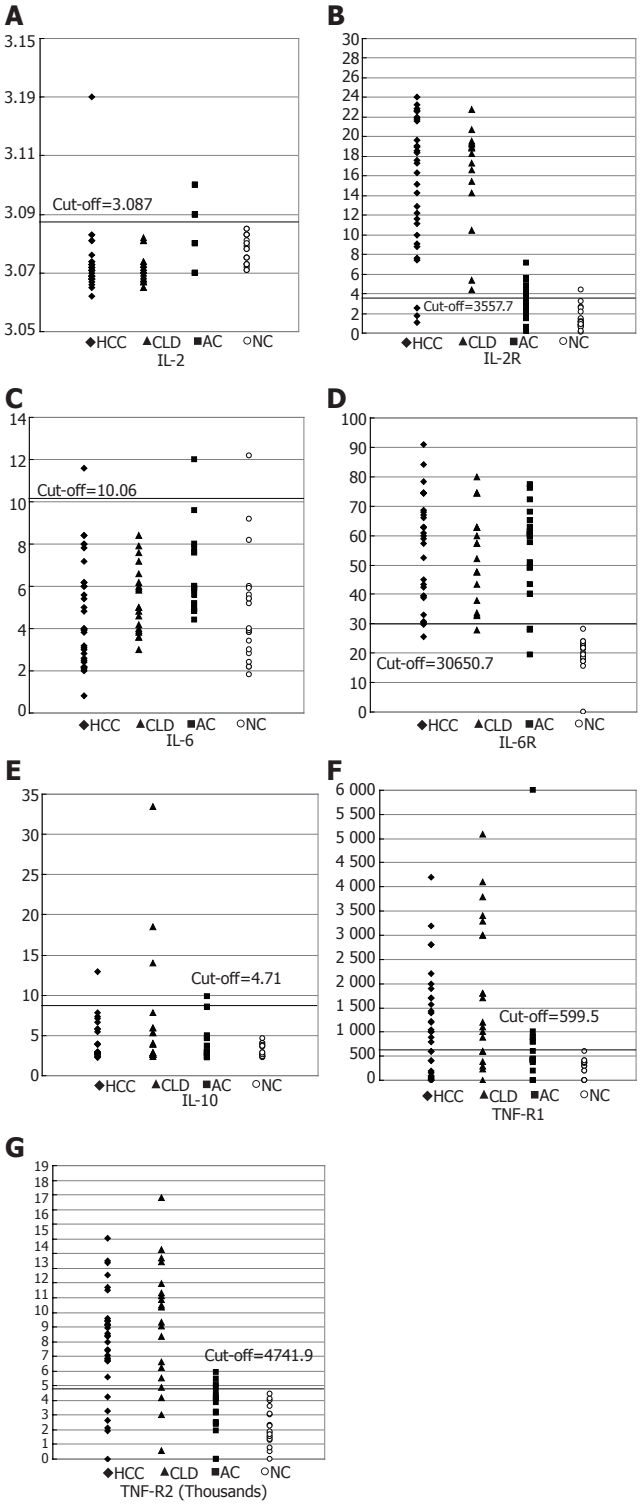

Figure 1 Scatter diagram represents the distribution value of (**A**) IL-2, (**B**) IL-2R (**C**) IL-6, (**D**) IL-6R, (**E**) IL-10, (**F**) TNF-R1, (**G**) TNF-R2 in HCC, CLD, ASC and normal controls around the cut-off value.

Table 1 Clinical features of study groups

Variables	HCC (n = 33)	CLD (n = 20)	ASC (n = 20)	NC (n = 20)	P
Age (mean±SD)	55.3±10.1	63.3±8.3	34±7.7	31.7±11.8	
Gender (M/F)	27/6	14/6	14/6	12/8	
Liver state:					
Cirrhosis	30 (91%)	14 (70%)	0	0	0.05
Irregular surface	30 (91%)	12 (60%)	0	0	0.007
Ascites	13 (39.4%)	11 (55%)	0	0	0.27
Jaundice	27 (81.8%)	8 (40%)	0	0	0.002
LL edema1	19 (57.6%)	11 (55%)	0	0	0.85
Megaly	33 (100%)	19 (95%)	0	0	0.38
Spleenomegaly	19 (57.6%)	13 (65%)	0	0	0.59
Schistosomal-Ab	18 (54.5%)	13 (65%)	14 (70%)	1 (5%)	0.45
HCV-Ab	31 (93.9%)	15 (75%)	20 (100%)	1 (5%)	0.05
HBsAg	3 (9%)	6 (30%)	4 (20%)	0	0.05
HCV-RNA	22 (66.7%)	14 (70%)	20 (100%)	0	0.8
Liver function tests					
ALT (mean±SD)	67.2±43.3	48±33.1	27.2±(7.0)	25.3±(4.2)	0.06
AST (mean±SD)	126.4±57.4	83.6±54.1	26.1±(5.0)	19.1±(5.0)	0.006
Alk ph (mean±SD)	260.4±193.4	221±126.7	70±26.1	65±24.2	0.414
Bil (mean±SD)	2.28±2.1	2.02±1.9	1.0±0.2	0.9±0.1	0.34

[1]LL edema: lower limb edema. P<0.05 difference is statistically significant.

Table 2 Serum levels of cytokines in the study groups (mean±SD)

Cytokine	HCC (n = 33)	CLD(n = 20)	ASC(n = 20)	NC(n = 20)	P
IL-2	3.07±0.01	3.07±0.005	3.07±0.009	3.08±0.004	>0.05
Cut-off=3.087 pg/mL					
Number of positive cases	1 (3%)	0	6 (30%)	0	
IL-2R	16.67±5.44	16.28±5.28	3.42±1.85	1.39±1.09	<0.001
Cut-off=3.5 ng/mL					
Number of positive cases	30 (91%)	16 (80%)	9 (45%)	1 (5%)	
IL-6	4.71±2.49	5.2±1.68	6.4±1.9	4.82±2.62	0.032
Cut-off=10.06 ng/mL					
Number of positive cases	1 (3%)	0	1 (5%)	1 (5%)	
IL-6R	56.48±17.97	54.47±16.72	54.13±16.35	19.62±5.52	<0.001
Cut-off=30.65 ng/mL					
Number of positive cases	29 (88%)	19 (95%)	17 (85%)	0	
IL-10	9±26.07	6.59±7.57	3.96±2.24	3.13±0.79	>0.05
Cut-off=4.71 pg/mL					
Number of positive cases	9 (31%)	8 (40%)	3 (15%)	0	
TNF-α	4.77±3.2	5.53±1.9	811±5.8	58±196	<0.001
Cut-off=17.2 ng/mL					
Number of positive cases	0	0	20 (100%)	1 (5%)	
TNF-α RI	1.27±0.98	1.87±1.50	0.56±0.23	0.29±0.16	<0.001
Cut-off=0.599 ng/mL					
Number of positive cases	24 (72%)	16 (80%)	7 (35%)	1 (5%)	
TNF-α RII	7.89±3.57	9.16±4.26	3.42±1.72	2.17±1.29	<0.001
Cut-off=4.74 ng/mL					
Number of positive cases	25 (81%)	17 (85%)	4 (20%)	0	

[1]Cut-off=mean±2 SD of non-cancer controls. P<0.05 difference is statistically significant.

But, in comparison to non-cancer controls, HCC patients had higher values of IL-2R, IL-6R, TNF-RI and TNF-RII, but less TNF-α (P<0.001). CLD patients had higher IL-2R, TNF-RI and TNF-RII (P<0.001) than in ASC. But, in comparison to non-cancer controls, CLD patients had higher IL-2R, TNF-RI and TNF-RII, but lower TNF-α (P<0.001). IL-10 was higher (though not significantly) in HCC and CLD patients than in symptomatic carriers and non-cancer controls. No significant difference was noticed in any of the clinical characteristics of patients with cytokines values above the cut-off, when compared with those below the cut-off in both HCC and CLD patients.

The most sensitive cytokines as markers for disease progression in HCV-infected patients were IL-2R (91% of HCC and 80% of CLD patients had values above the cut-off), IL-6R (88%, 95%, and 85% of HCC, CLD and ASC respectively, had values above the cut-off) and also TNF-RII (81% of HCC and 85% of CLD cases had values above the cut-off).

DISCUSSION

It has been reported that hepatotropic viruses HBV, HDV, HCV are associated with HCC, and more than 80% of HCCs that occur worldwide are thought to be associated with chronic viral hepatitis[10]. In our series, the prevalence of anti-HCV antibodies by the third-generation ELISA was 93.9% in HCC patients. HCV-AB

was positive in 86.5% of 37 HCC patients in our previous study[11]. However, this ratio is higher than those reported by other researchers[12,13]. The present study showed that the prevalence of anti-HCV antibodies was 75% in CLD patients, which is also higher than those reported by other researchers[14,15].

The HCV viremia in our HCV-serpositive HCC cases was also higher than those reported by other researchers[11,16,17]. This discrepancy might be explained by the fluctuations of the amount of the viruses in the serum[18]; or the discrepancy of RT-PCR methods used by the different series[8].

In our study, HBsAg was found in 9% of HCC cases, which is similar to that in our previous studies[11,12]. HBsAg was found in 30% of our CLD cases, which is higher than that reported by Angelico *et al*.[14]. In the present study, history of bilharziasis and prior anti-bilharzial parenteral therapy was found in 51.6% and 41.9% of HCC patients and in 73.3% and 60% of CLD patients respectively, indicating that history of parenteral anti-schistosomal therapy is a major risk factor for transmitting HCV infection.

Anti-bilharzial antibodies were found in 55% of HCC patients in our study, which is higher than that reported in our previous studies[11]. This high incidence could be attributed to the fact that most of our HCC patients (66.7%) came from rural areas. Rural populations in both Egypt and other African areas have a higher incidence of viral hepatitis. In the rural areas, many individuals may become infected due to tattooing[19].

A characteristic feature of HCV infection is a high frequency of persistence and progression to CLD. Persistent infection upsets the balance between immunostimulatory and inhibitory cytokines, which can prolong inflammation and lead to necrosis, fibrosis, and CLD[1]. Elevated concentrations of cytokines also represent a characteristic feature of CLD, regardless of underlying etiology, which may represent a consequence of liver dysfunction instead of inflammatory disorder[20].

Missale *et al*.[2] and Cacciarelli *et al*.[3] found that serum IL-2 is significantly elevated in HCV chronically HCV-infected patients. However, Simsek and Kadayifci[21] found that serum IL-2 has no significant change in the same group of patients. In contrast, our study showed no significant change in the levels of IL-2 among the different groups. The high level of sIL-2R might explain the apparently normal level of IL-2 in our patients as low IL-2 level could therefore be due to soluble receptor binding to IL-2. This theory is also supported by Sismek and Kadayifci[21]. On the other hand, Izzo *et al*.[22] found that serum levels of sIL-2R correlate with the histological severity of liver damage in patients with chronic HCV infection and may be used as a marker in patients at high risk of getting HCC and the highest levels of soluble IL-2R occur in patients with HCC[23]. We could not, however, correlate serum levels of sIL-2R with the different clinical and biochemical findings in HCC in this study.

Thus, sIL-2R may play a role in the pathogenesis of chronic active hepatitis and HCC, and could be considered as a good marker for disease progress in chronic HCV-infected patients. IL-2R was above normal in 91% of HCC and in 80% of CLD cases in our study.

Interleukin-6, a multifunctional cytokine produced by a variety of cells, plays a central role in regulating the immune system, hematopoiesis, and acute phase reaction. It interacts with a receptor complex consisting of a specific ligand-binding protein (IL-6R, gp80) and a signal transduction protein (gp130)[24,25]. Serum IL-6 levels are higher in patients with chronic HCV infection in comparison to healthy adults[26,27]. In contrast, our results showed that IL-6 was slightly higher only in asymptomatic HCV carriers than in non-cancer controls, but apparently normal in both HCC and CLD patients, which is in accordance with that reported by Tovey *et al*.[28]. Altered IL-6 gene expression is a characteristic feature of advanced stage of severe liver disease. McGuinness *et al*.[29] also found that IL-6 mRNA is down-regulated in chronic HCV-infected patients.

On the other hand, Steffen *et al*.[30] revealed that IL-6 is inversely correlated with sIL-6R, as IL-6 may play a role in the decrease of sIL-6R either by the direct inhibitory effect on the expression of the IL-6R gene or by the formation of IL-6R/sIL-6R complexes followed by their internalization in target cells. This might partially explain the apparently normal levels of IL-6 in our HCC and CLD patients by the increase in their sIL-6R levels.

The pro-inflammatory cytokine tumor necrosis factor-α plays an important role in the pathophysiology of liver disease. Specific antagonists of this cytokine have been found in recent years. TNF soluble receptors p55 and p57 derived from the cell surface are naturally occurring substances that inhibit the biological effects of tumor necrosis factor.

The striking elevation of pro-inflammatory cytokine TNF-α in asymptomatic HCV carriers may reflect both insufficiency of HCV elimination and a failure to control the cytokine cascade. Our result is opposite to those reported by Goyal A[20] and Toyoda *et al*.[31]. This could be attributed also to the difference in the epitopes of the ELISA system used by the different groups or to the difference in genotypes. All our cases showed HCV genotype-4.

The promoter in the IL-6 gene has been shown to contain regulatory regions to which DNA-binding proteins can bind. These DNA-binding proteins, induced by IL-1 and TNF-α, stimulate transcription of the IL-6 gene. Thus, as IL-1 and TNF-α levels increase, production of IL-6 also increases[32]. Our result is in accordance with the above findings, and the decrease in TNF-α is associated with a decrease in IL-6 in HCC and CLD cases.

In our study, HCC cases had significantly higher values of TNF-RII and TNF-RII ($P<0.001$) than ASC and NC subjects. Thus, the rise in concentrations of TNF receptors I and II in our patients suggests that HCV-related liver disease involves immunological mechanisms including activation of the TNF system and may reflect the degree of inflammation and development of HCC. These results are in accordance with other studies[33,34].

Tai *et al*.[5] also showed that sTNF-RI levels correlate with liver inflammation in all patients, whereas this correlation cannot be found with sTNF-RII, IL-2, IL-10 and TNF-α.

Accordingly, we can use TNF-RII as a marker in HCV-infected cases at high risk of getting CLD and HCC.

Delpuech *et al*. [35] showed that even if the infection of H9 T cell line with HCV does not result in any viral progeny, HCV induces the activation of IL-10 secretion, which supports the role of IL-10 in HCV pathogenesis. Cacciarelli *et al*.[3] and Kakumu *et al*.[33] found that serum IL-10 levels are significantly higher in all CLD groups than in controls, indicating that IL-10 reflects the degree of inflammation in the liver and may be related to the development of HCC. In our series, IL-10 levels were increased (though not significantly) in HCC, CLD in comparison to non-cancer controls. The positive HCV-viremic CLD cases also had higher values of IL-10 ($P = 0.08$) than non-viremic cases. In addition to the production at the site of inflammatory changes with activated infiltrating mononuclear cells in the liver, the high serum IL-10 levels in patients with HCC presumably also result from the secretion of IL-10 by tumor cells. Its secretion by human hepatocellular tumors has been observed previously[36]. Thus, a high IL-10 level is suggested to contribute to a relative state of immuno-suppression, and in patients with HCC, may help the tumor cells escape host immune surveillance and potentiate tumor cells to metastasize[37].

It has been postulated that an imbalance between Th1 and Th2 cytokine production is implicated in disease progression or inability to clear infections. It was reported that HCV-infected patients who develop chronicity have a predominant Th2 response, but a weak Th1 response, suggesting that this immune response imbalance can result from HCV interaction with dendritic cell functions[38]. These results agree with ours and support the notion that Th-lymphocyte polarization may play an important pathophysiologic role in influencing the outcome of HCV infection. All these immunological findings are mostly due to HCV infection rather than schistosomal infection, because patients with no schistosomal antibody had the same elevation of the same cytokines, late Schistosoma mansoni cases showed a suppressed cell-mediated immunity and a significant depletion of T-helper/inducer subset[39].

In conclusion, the most sensitive cytokines as markers for disease progression in HCV-infected patients are IL-2R, IL-6R and TNF-RII. Accordingly, we may use serum IL-2R, IL-6R, TNF-RII as markers in HCV-infected cases at high risk of getting CLD and HCC. Thus, disease progression due to HCV infection is associated with decrease of circulating Th1 cytokines (IL-2) and increase of Th2 cytokine (IL-10). Persistent infection upsets the balance between immunostimulatory and inhibitory cytokines, which can prolong inflammation and lead to necrosis, fibrosis, and CLD.

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Toll-like receptor 4 plays an anti-HBV role in a murine model of acute hepatitis B virus expression

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Abstract

AIM: Toll-like receptor 4 (TLR4) has been shown to be important for bacterial infection, especially to lipopolysaccharide signaling. Its possible role in HBV infection is studied in the present study.

MATERIALS AND METHODS: pHBV3.6 plasmid, containing full-length HBV genome was used in the murine model of acute HBV expression by hydrodynamics *in vivo* transfection. TLR4 normal or mutant mouse strain was compared to investigate the possible role of TLR4 in acute HBV expression.

RESULTS: After pHBV3.6 injection, the infiltrating leukocytes expressed TLR4 were observed nearby the HBsAg-expressing hepatocytes. The HBV antigenemia as well as the replication and transcription were higher in TLR4-mutant C3H/HeJ mice than in normal C3H/HeN mice. The HBV-specific immune responses were impaired in the liver or spleen of the C3H/HeJ mice. Their inducible nitric oxide synthase (iNOS) expression on the hepatic infiltrating cells was also impaired. When adoptively transferring splenocytes from C3H/HeN mice to C3H/HeJ mice, the HBV replication was inhibited to the level as that of C3H/HeN.

CONCLUSION: These results suggest that TLR4 plays an anti-HBV role *in vivo* through the induction of iNOS expression and HBV-specific immune responses after HBV expression.

Key words: TLR4; Rodent; HBV; iNOS; Liver

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INTRODUCTION

Hepatitis B virus (HBV) is an enveloped, double-strand DNA virus, and its replication is through an RNA intermediate that requires reverse transcriptase activity[1]. HBV infection in human beings can cause chronic hepatitis and is associated with liver cirrhosis and hepatocellular carcinoma[2,3]. One-third of the global population has been infected with HBV and about 350 million people are chronic carriers of HBV[4]. HBV is non-cytopathic to hepatocytes and the hepatitis it causes is thought to be mediated by immune mechanism. However, the innate immune response to HBV infection is not fully understood.

Toll receptors are type I membrane proteins that was first identified in Drosophila and play a key role in antifungal immunity of Drosophila[5]. The mammalian homologs of Drosophila Toll protein are called Toll-like receptors (TLRs), and there are 10 human (TLR1 to TLR10) and murine TLRs (TLR 1 to TLR9 and TLR11)[5,6]. TLRs play a key role in host defense against microbial infection by regulating both innate and acquired immunity[7,8]. For example, TLR4 is the receptor of Gram-negative bacterial lipopolysaccharide (LPS). After binding, the MyD88, interleukin-1 receptor-associated kinase, and tumor necrosis factor receptor associated factor 6 are activated, and then through MAP kinases and NF-κB transcription factors[6,9] to turn on the genes expression, which were involved in the inflammatory responses[10]. TLR4 also plays a role in viral infections. Respiratory syncytial virus (RSV) persists longer in the lung of TLR4-deficient mice than normal mice, and RSV fusion protein can activate the human monocytes through TLR4[11].

Hydrodynamics-based *in vivo* transfection has been

recently described. With this procedure, naked DNA can be introduced and expressed significantly in liver[12,13]. This property allows investigators to develop hepatitis virus infection model in mouse[14]. Recently, a murine acute HBV expression model was generated by hydrodynamics-based injection of plasmid containing full-length HBV genome by our group[15] or others[16]. After hydrodynamic injection of pHBV3.6, including full-length HBV genome, the HBV transcript and replicative intermediate were induced in the liver whereas the HBV-antigens, HBV-DNA, and HBV-specific antibody were detected in the sera[15].

We are interested in the role of TLR4 during HBV infection. Using the murine model of acute HBV expression in this study, we reported that HBV expression-induced TLR4 expression has anti-HBV activity by upregulating the iNOS expression and HBV-specific immune response to help clearing the virus.

MATERIALS AND METHODS

Mice

Breeder mice of C3H/HeN and C3H/HeJ strain were purchased from The Jackson Laboratory (Bar Harbor, ME, USA) or Charles River Japan, Inc. (Atsugi, Japan). They were fed standard laboratory chow and water *ad libitum* in the animal facility. The animals were raised and cared for according to the guidelines set up by the National Science Council of the Republic of China. Eight- to twelve-week-old male mice were used in all experiments.

Plasmids

pHBV3.6 containing all HBV open-reading frames was provided by Dr LP Ting (Department of Microbiology and Immunology, National Yang-Ming University), p(3A)SAg that encodes HBsAg was provided by Dr CC Lu (Department of Pathology, National Cheng Kung University) and pHBV^PSX that encodes HBcAg was provided by Dr SJ Lo (Department of Microbiology and Immunology, National Yang-Ming University). pEGFP-N1 was obtained from Clontech (Palo Alto, CA, USA). All plasmids were prepared with Hi-speed Plasmid Midi Kit (Qiagen, Hilden, Germany).

Cells and transfection

The C3H/He bladder cancer cell line, MBT-2, was kindly provided by Dr MD Lai (Department of Biochemistry, National Cheng Kung University). Cells were maintained in Dulbecco's modified Eagle medium (Gibco BRL, Grand Island, NY, USA) and 10% fetal bovine serum (HyClone, Logan, UT, USA) at 37 ℃ under 50 mL/L CO_2. The cells were plated at a density of 3×10^5 cells/well in six well-culture plate. One day later, cells were transfected with 1 μg of p(3A)SAg and pHBV^PSX using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. The medium was replaced with a fresh medium 8 h after transfection, and cells were used for T cell stimulation at 36 h after transfection.

Hydrodynamics-based in vivo transfection

Ten micrograms of plasmid, dissolved in Ringer's solution (NaCl 0.154 mol/L, KCl 5.63 mmol/L, $CaCl_2$ 2.25 mmol/L), were injected in the mouse tail vein, within 5 to 7 s, at a 12% of mouse bodyweight (around 3.0 mL) following the hydrodynamics-based transfection protocol described previously[15].

Immunohistochemical analysis of HBsAg, TLR4, and iNOS expression

Mouse liver tissues were embedded in OCT compound (Miles Inc., Elkhart, IN, USA) and frozen in liquid nitrogen. Four micrometer cryosections were made using cryostats (Leica CM 1800, Nussloch, Germany). For TLR4 and HBsAg double staining, sections were fixed by 3.7% formaldehyde/PBS and then firstly stained with sheep-anti-HBsAg antibody (Serotec, Oxford, UK) and FITC-conjugated donkey-anti-sheep antibody (Jackson Laboratories, West Grove, PA, USA). After washing with PBS, the sections were further stained with rat-anti-mouse TLR4 antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) and Rhodamine-conjugated donkey-anti-rat antibody (Jackson Laboratories, West Grove, PA, USA). The nucleuses were stained by Hoecsht 33258. For HBsAg and iNOS staining, the sections were fixed by cold acetone and endogenous peroxidase was inhibited by 3% H_2O_2/PBS. HBsAg and iNOS were detected with sheep polyclonal anti-HBs (Serotec, Oxford, UK) and rabbit polyclonal anti-iNOS antibodies (Chemicon, Temecula, CA, USA), respectively. Secondary antibody used was either biotinylated anti-sheep or anti-rabbit, and then incubated with avidin-peroxidase complex (Vector Laboratories, Burlingame, CA, USA). Peroxidase stain of red color was developed by aminoethyl carbazole substrate (Zymed Laboratories, San Francisco, CA, USA) and counterstained with Mayer's hematoxylin (Merck, Darmstadt, Germany).

Northern and Southern blot hybridization of HBV transcription and replication

Total RNA was purified from mouse liver by TRI Reagent (Molecular Research Center, Inc, Cincinnati, OH, USA). The cytoplasmic DNA of mouse liver was purified as described previously by Guidotti *et al*.[17]. Thirty micrograms of total RNA or cytoplasmic DNA isolated from 30 mg of liver tissue were run on agarose gel electrophoresis, transferred to a nylon membrane and hybridized with biotin labeled HBV specific DNA probe, which was prepared by PCR amplication with the primers as: HBV1806: 5'-CCGGAAAGCTTGAGCT CTTCAAAAAGTATGGTGCTGG-3' ; HBV1821: 5' -CCGGAAAGCTTCTTTTTCACCTCTGCCTAATCA-3', at 45 ℃ overnight. To verify the transfection efficiency, we injected pEGFP-N1 with pHBV3.6 simultaneously and detected by a specific biotin labeled DNA probe. The hybridized bands were detected by Detector AP Chemiluminescent Blotting Kit (KPL, Inc., Gaithersburg, MD, USA) and visualized by X-ray films.

HBV DNA detection in mouse sera

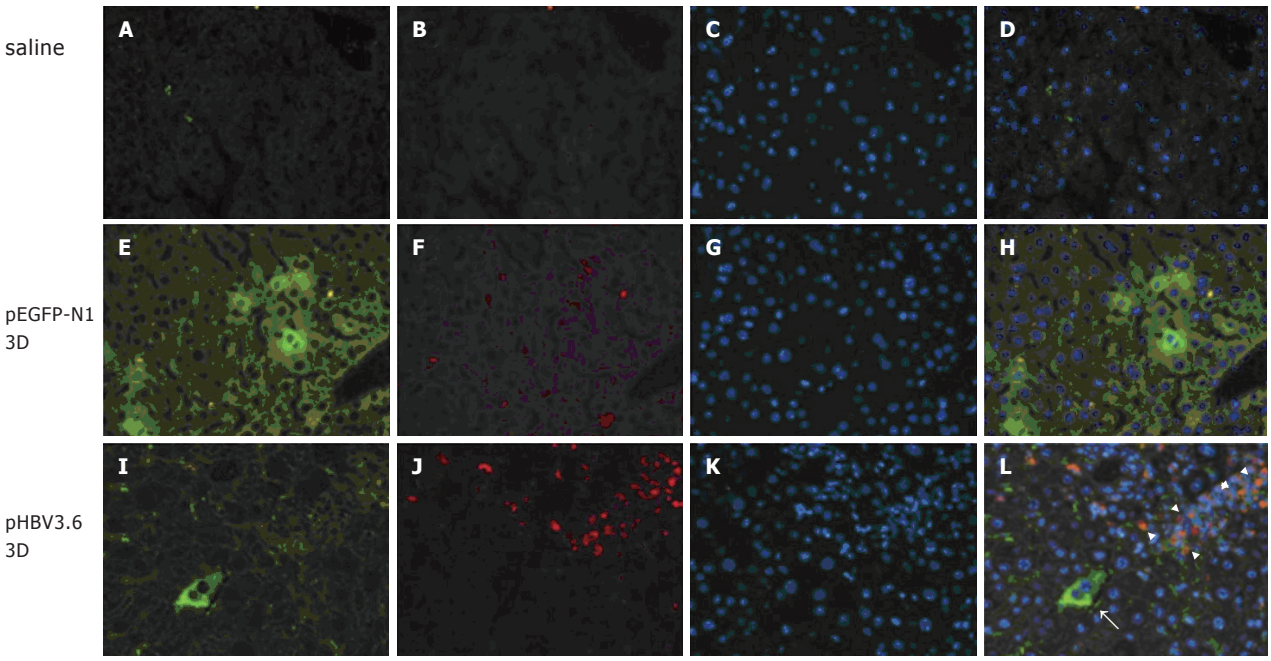

Figure 1 TLR4 expression in the liver of C3H/HeN mice after hydro-dynamic injection of pHBV3.6. Groups of four C3H/HeN mice were injected intravenously with 10 μg of plasmid by hydrodynamics-based transfection. The liver tissues were collected at day 3 post injection and 4-μm cryosections were made, stained with anti-HBsAg-FITC and anti-TLR4-PE antibody. The green represents EGFP-positive (**E**) or HBsAg-positive cells (**I**) and the red represents TLR4-positive cells (**B, F, J**). The blue represents counter staining by Hoechst 33258 dye (**C, G, K**). **A-D:** naïve; **E-H:** pEGFP-N1; **I–L:** pHBV3.6. The arrows indicate the HBsAg positive hepatocytes and the arrowheads indicate the TLR4 positive immune cells (original magnification ×200)

Two hundred microliters of mouse serum was treated with 20 U DNase I for at least 12 h to remove retaining plasmid. After DNase I treatment, the serum DNA was purified by Viral DNA/RNA Isolation Kit (Maxim Biotech, INC., San Francisco, CA, USA). Five microliters of isolated DNA solution was used to detect the HBV DNA by PCR analysis. The preS2 region of surface antigen gene was amplified and visualized on an agarose gel as described previously[18].

Detection of HBsAg and HBeAg in mouse sera

The level of HBsAg and HBeAg were determined using enzyme-linked immunosorbent assay (ELISA) kits (General Biological Corp., Taiwan, ROC) following the manufacturer's protocol.

ELISA for detecting cytokines

The intrahepatic lymphocytes (IHLs) were isolated as described previously[15] with further removing the adhering cells. The IHLs or splenocytes were co-cultured with mitomycin C-treated (100 μg/mL at 37 ℃ for 90 min) MBT-2[19] or p(3A)SAg and pHBV^PSX transfected, MBT-2 (MBT-2-SC) at a ratio of 10:1 for 48 h The co-cultured supernatants were harvested and assayed by sandwich ELISA for mouse IFN-γ, TNF-α or IL-12 (R&D Systems, Minneapolis, MN, USA).

RESULTS

Infiltrating cells in the liver expressed TLR4 after transfection of HBV gene

To investigate the association of TLR4 and HBV expression, the plasmid pHBV3.6, containing the full-length HBV genome was administrated to C3H/HeN mice by hydrodynamics *in vivo* transfection. As shown in Figure 1, the TLR4 expression could be detected on infiltrating leukocytes at day 3 post-injection (Figures 1I-L). But it was not detected in the liver of naïve C3H/HeN mice (Figures 1A-D) or pEGFP-N1 injected mice (Figures 1E-H). These results indicate that TLR4 expressed on leukocytes might involve the acute HBV expression.

HBV antigenemia and replication was higher in C3H/HeJ than in C3H/HeN

In C3H/HeJ mice, a missense mutation in the cytoplasmic domain of TLR4 impaired its ability to respond to lipopolysaccharide (LPS)[20]. To further investigate the role of TLR4 in HBV expression, we compared the response in C3H/HeN (TLR4 normal) and C3H/HeJ (TLR4 mutation) after the pHBV3.6 *in vivo* transfection. As shown in Figure 2, the serum HBsAg (Figure 2A) or HBeAg (Figure 2B) level was higher in C3H/HeJ than in C3H/HeN mice. By Northern and Southern blot analyses, the specific HBV transcripts or HBV replication fragments were also higher in the liver of C3H/HeJ than C3H/HeN mice under similar transfection efficiency that was verified by co-injection of pEGFP-N1 (Figure 2C). Using viral DNA isolation and PCR analysis, the serum HBV-DNA showed higher level in C3H/HeJ than in C3H/HeN mice (Figure 2D). These results suggest that TLR4 is involved

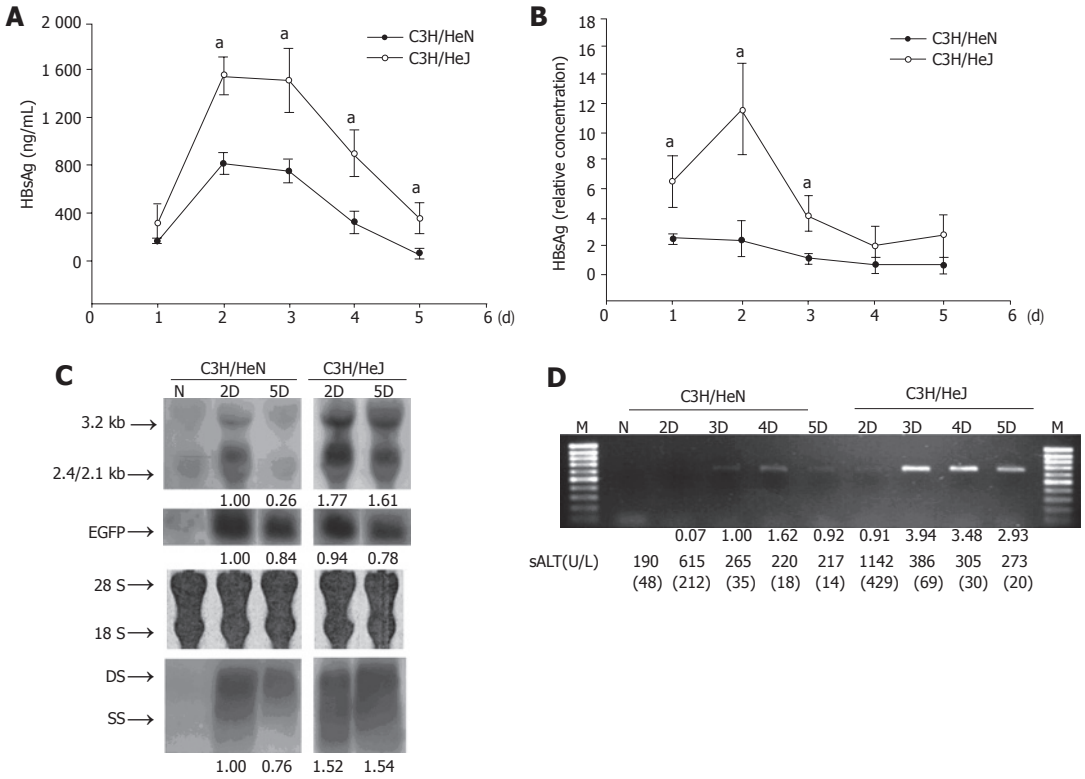

Figure 2 HBV antigenemia and replication in TLR4-mutant C3H/HeJ mice or normal C3H/HeN mice. Groups of four C3H/HeN or C3H/HeJ mice were injected intravenously with 10 μg of pHBV3.6 and 10 μg of pEGFP-N1 that was used as control of transfection efficiency by hydrodynamics-based transfection. The HBsAg (**A**) or HBeAg (**B**) in the serum was detected by ELISA kits as described in Materials and methods. [a]*P*<0.05. (**C**) The total RNA or cytoplasmic DNA was purified from liver and hybridized with HBV specific probes to analyze the HBV transcription or replication. The number below each land of Northern or Southern blot was represented as the relative fold of expression comparing to d2 result of C3H/HeN mice. N, naïve C3H/HeN. (**D**) The serum DNA was purified after treatment with 20 U DNase I and the HBV-DNA was detected by PCR method. The number below each land was represented as the relative fold of expression comparing to d3 result of C3H/HeN mice. M, 100-bp DNA ladder; N, naïve C3H/HeN. The sALT level was represented as mean (SD).

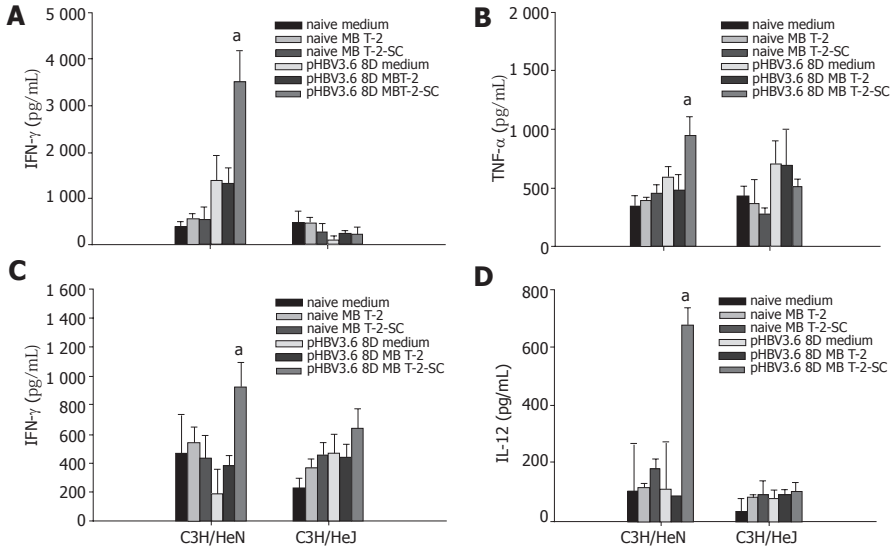

Figure 3 HBV-specific immune responses in the liver or spleen of C3H/HeN or C3H/HeJ mice after hydrodynamic-injection of pHBV3.6. Groups of four C3H/HeN or C3H/HeJ mice were injected intravenously with 10 μg of pHBV3.6 by hydrodynamics-based transfection. The intrahepatic lymphocytes (**A** and **B**) or splenocytes (**C** and **D**) were isolated at d 8 post injection. After being co-cultured with HBsAg and HBcAg expressing syngenic MBT-2 cells (MBT-2-SC) for 48 h, the IFN-γ (**A** and **C**), TNF-α (**B**) or IL-12 (**D**) in the culture supernatant was analyzed by ELISA method. [a]*P*<0.05 *vs* naïve MBT-2-SC group.

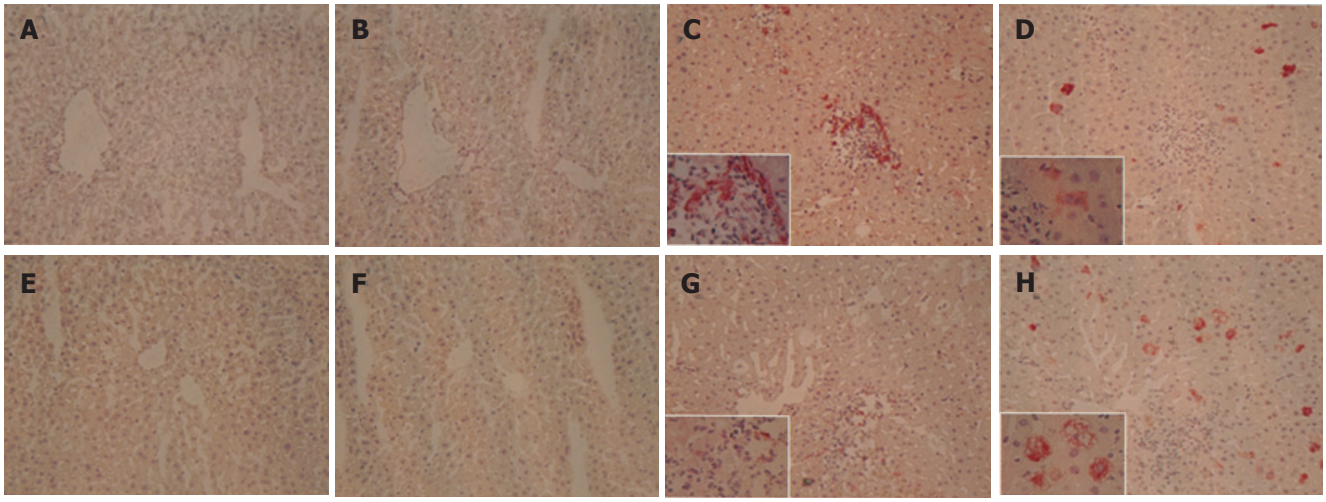

Figure 4 iNOS expression in the liver of C3H/HeN or C3H/HeJ mice after hydrodynamic-injection of pHBV3.6. Groups of four C3H/HeN (**C, D**) or C3H/HeJ (**G, H**) mice were injected intravenously with 10 μg of pHBV3.6 by hydrodynamics-based transfection. Serial sections of frozen liver tissue were made and stained with anti-HBs antibody (**B, D, F, H**) or anti-iNOS antibody (**A, C, E, G**). Red color was developed and indicated the positive staining. The insets in (**C**), (**D**), (**G**), and (**H**) represent twofold magnification of positive staining. **A, B, E, F,** saline injected control mice; **C, D, G, H,** day 2 post injection (original magnification ×200).

in host responses to HBV replication and a mutation in C3H/HeJ impaired its ability to clear the HBV virus.

HBV-specific immune responses were defective in TLR4 mutant C3H/HeJ mice after hydrodynamic injection of pHBV3.6

To investigate the effect of TLR4 on immune responses to HBV, intrahepatic lymphocytes (IHLs) or splenocytes from pHBV3.6-injected C3H/HeN or C3H/HeJ mice were isolated and stimulated with HBsAg- and HBcAg-expressing syngenic MBT-2 cells. The IFN-γ and TNF-α production of IHLs from C3H/HeN mice was significantly increased at 48 h after HBsAg and HBcAg stimulation. But this was not observed in IHLs from C3H/HeJ mice (Figures 3A and B). The IFN-γ and IL-12 production of splenocytes of C3H/HeN mice was also significantly increased in C3H/HeN mice, but not in C3H/HeJ mice (Figures 3C and D). These data suggest that TLR4 mutation in C3H/HeJ affects the HBV specific immune responses (IFN-γ and TNF-α production in liver or IFN-γ and IL-12 production in spleen).

Induction of iNOS was also defective in TLR4 mutant C3H/HeJ mice after hydrodynamic injection of pHBV3.6

It was reported that the activation of TLR4 signaling can induce the iNOS expression[21], and we also reported that iNOS plays an anti-HBV role in acute HBV expression[15]. Therefore, the iNOS expressed was compared in wild type and TLR4 mutant mice after pHBV3.6 transfection. The HBsAg-expressing hepatocytes were equivalent among C3H/HeN and C3H/HeJ mice after pHBV3.6 injection, indicating the transfection efficiency was similar in both strains of mice. But iNOS stainings were detected on the infiltrating leukocytes nearby the HBsAg-expressing hepatocytes in the liver of pHBV3.6-injected C3H/HeN (Figures 4C and D) whereas the expression of iNOS on infiltrating leukocytes was impaired in the liver of C3H/HeJ mice (Figures 4G and H). These data suggested that the mutation of TLR4 influences the induction of iNOS expression during acute HBV expression and may further affect the clearance of HBV.

HBV replication was reduced in C3H/HeJ mice after adoptive transfer of splenocytes from C3H/HeN mice

To further confirm, if TLR4-expressing immune cells provide the protection role to HBV expression, the splenocytes from C3H/HeN mice were injected into C3H/HeJ mice intravenously at the day before pHBV3.6 injection. The HBsAg antigenemia was higher in C3H/HeJ than in C3H/HeN mice, but the adoptive transfer of C3H/HeN splenocytes into the C3H/HeJ mice reduced the serum HBsAg to the level similar to that in C3H/HeN mice (Figure 5A). The HBV transcription or replication in liver (Figure 5B) or HBV-DNA in sera (Figure 5C) was also reduced as well under similar transfection efficiency as verified by co-injection of pEGFP-N1. But when adoptive transfer of splenocytes from C3H/HeJ mice to C3H/HeN mice was carried out, there was no effect to HBsAg in sera (Figure 5A), HBV replication in liver (Figure 5B), or HBV-DNA in sera (Figure 5C). These results indicate that the TLR4 enhances the immune functions to help clear the HBV replication.

DISCUSSION

In this study, we investigated the role of TLR4 in a murine acute HBV expression model. The HBV antigenemia, serum HBV-DNA, as well as HBV transcription and replication in liver were higher in TLR4 mutant C3H/HeJ than TLR4 normal C3H/HeN. This is probably caused by the impaired induction of iNOS and HBV specific immune response because of TLR4 mutation in C3H/

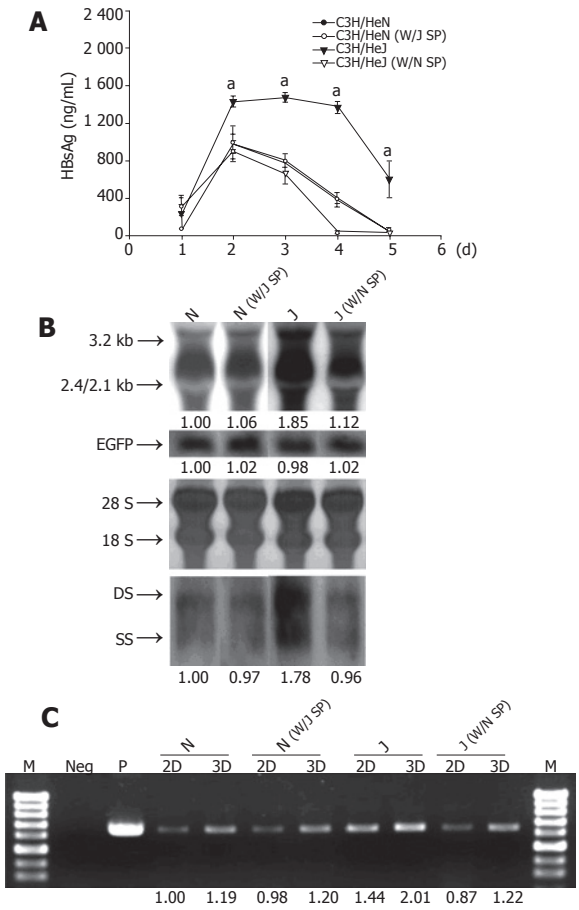

Figure 5 HBV replication was reduced in C3H/HeJ mice after adoptive transfer of splenocytes from C3H/HeN mice. 5×10^6 splenocytes from C3H/HeN or C3H/HeJ mice were injected into C3H/HeJ or C3H/HeN mice intravenously at the day before pHBV3.6 injection, respectively. (**A**) The HBsAg in sera was detected as indicated day by ELISA. $^aP<0.05$. (**B**) The total RNA or cytoplasmic DNA of liver was isolated at day 3 and was further analyzed for HBV transcription or replication by Northern or Southern blot. The transfection efficiency was verified by co-injection of pEGFP-N1 and EGFP RNA confirmation. The number below each land of Northern or Southern blot was represented as the relative fold of expression comparing to C3H/HeN mice. N, C3H/HeN; J, C3H/HeJ; SP, splenocytes. (**C**) The HBV-DNA in sera was isolated as on the indicated day and detected by PCR method. The number below each land was represented as the relative fold of expression comparing to day 2 result of C3H/HeN mice. M, 100-bp DNA ladder; Neg, no template control; P, pHBV3.6 5 ng; N, C3H/HeN; J, C3H/HeJ; SP, splenocytes.

HeJ mice, and suggests that TLR4 plays an antiviral role in HBV replication. Recently, Isogawa *et al*. reported that TLR signaling which includes TLR4 could inhibit HBV replication in transgenic mice model and the mechanism might be through the induction of type I interferon[22]. Our finding of iNOS and HBV specific immune response has increased further understanding on anti-HBV response in addition to the induction of type I interferon.

The TLRs are pattern-recognition receptors that have an important role in mammalian immunity[7,8]. At least 10 TLRs are expressed on a variety of cell types including immune cells, endothelial cells[23], cardiac myocytes[24], and intestinal epithelial cells[25]. Most TLR ligands are conserved pathogen-associated molecular patterns of microbes and the TLR signals serve as a sensor to the presence of infection[13]. TLR4 is the first identified mammalian TLR that expresses predominantly on macrophages and dendritic cells (DCs)[5]. A point mutation ($His^{712}\rightarrow Pro^{712}$) in the Toll/interleukine-1 receptor domain of TLR4 gene causes the C3H/HeJ mice to become defective after LPS challenge[20,26]. TLR4 has been shown to initiate a response to the fusion protein of RSV[11]. Prolonged RSV infection was found in TLR4-deficient mice because of the impaired recruitment of natural killer cells and $CD14^+$ cells in the lung tissue as well as the impaired IL-12 production[27]. Two vaccinia virus ORFs, A46R, and A52R, have been shown to share amino acid sequence similarity to TIR domain, and these two proteins can partially or potentially inhibit the IL-1 and TLR4 mediated NF- κ B activation[28]. These reports indicate that TLR4 also has anti-virus activity in addition to its anti-bacterial function.

In our study, the IHLs nearby the HBsAg-positive hepatocytes (which represented HBV-replicating hepatocytes) expressed TLR4 post pHBV3.6 hydrodynamic transfection (Figure 1). The HBV replication was higher in TLR4 mutant C3H/HeJ than C3H/HeN mice (Figure 2). This is probably caused by the impaired specific anti-HBV immunity in liver or spleen (Figure 3). When adoptively transferring splenocytes from TLR4 normal C3H/HeN mice to TLR4 mutant C3H/HeJ mice, the anti-HBV responses could be upregulated (Figure 5). These results indicate that anti-HBV specific immune responses may be initiated by TLR4 activation. Activation of DCs by LPS through TLR4 has been found to induce IL-12 production and elicit the Th1 responses against intracellular pathogens[29]. In our study, the IL-12 production in splenocytes after HBsAg or HBcAg stimulation (Figure 3D) and IFN-γ production in IHLs (Figure 3A) was also impaired in the C3H/HeJ mice. These results correlated with the impaired function of TLR4 in the C3H/HeJ mice.

TLR4 can signal to induce iNOS expression. After TLR4 agonist stimulation, iNOS gene could be induced through MyD88-dependent (NF-κB) or -independent (IFN-β and STAT-1) pathway in murine macrophage cell line[21]. We have also reported the role of iNOS in anti-HBV response[15]. The iNOS induction in the infiltrating immune cells was impaired in C3H/HeJ mice (Figure 4). Therefore, the iNOS may also involve in the TLR4-mediated anti-HBV responses. In conclusion, the present study reports that TLR4 plays an anti-HBV role in acute HBV expression through induction of iNOS expression and specific anti-HBV immune responses.

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He Jie Tang in the treatment of chronic hepatitis B patients

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Abstract

AIM: To explore the effect of He Jie Tang (decoction for medication) on serum levels of T lymphocyte subsets, NK cell activity and cytokines in chronic hepatitis B patients.

METHODS: Eighty-five patients with chronic hepatitis B were divided randomly into two groups. Fifty patients in group I were treated with He Jie Tang (HJT) and 35 patients in group II were treated with combined medication. The levels of T-lymphocyte subsets (CD_3^+, CD_4^+, CD_8^+), NK cell activity, cytokines (TNF-α, IL-8, sIL-2R) were observed before and after the treatment. Another 20 normal persons served as group 3.

RESULTS: The level of CD_4^+ cells and NK cell activity were lower, whereas the level of CD_8^+ cells in patients was higher than that in normal persons (t = 2.685, 3.172, and 2.754 respectively; P<0.01). The levels of TNF-α, IL-8, and sIL-2R in chronic hepatitis B patients were higher than those in normal persons (t = 3.526, 3.170, and 2.876 respectively; P<0.01). After 6 months of treatment, ALT, AST, and TB levels in the two groups were obviously decreased (t = 3.421, 3.106, and 2.857 respectively; P<0.01). The level of CD_4^+ cells and NK cell activity were increased whereas the level of CD_8^+ cells decreased (t = 2.179, 2.423, and 2.677 respectively; P<0.05) in group I. The levels of TNF-α, IL-8, and sIL-2R in group I were decreased significantly after the treatment (t = 2.611, 2.275, and 2.480 respectively; P<0.05) but had no significant difference in group II after the treatment (t = 1.906, 1.833, and 2.029 respectively; P>0.05). The total effective rate had no significant difference between the two groups (χ^2 = 2.882, P>0.05) but the markedly effective rate was significantly different between the two groups (χ^2 = 5.340, P<0.05).

CONCLUSION: HJT is effective in treating chronic hepatitis B. HJT seems to exert its effect by improving the cellular immune function and decreasing inflammatory cytokines in chronic hepatitis B patients. The function of HJT in protecting liver function in the process of eliminating virus needs to be further studied.

Key words: He Jie Tang; Lymphocyte subsets; NK cell; Cytokines; Chronic hepatitis B

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INTRODUCTION

Chronic hepatitis B virus (HBV) infection is a serious clinical problem worldwide and may lead to end-stage liver disease, cirrhosis, and hepatocellular carcinoma (HCC), *etc.*[1-3]. The pathogenesis of hepatitis B is very complex and has not been clarified. Generally, HBV itself does not directly damages hepatocytes, but results in dysfunction of cell-mediated immunity[3-5]. Peripheral blood mononuclear cells (PBMCs), which are aggregated immunologically competent cells, such as T lymphocytes, natural, and lymphokine-activated killer cells, likely play an important role in anti-HBV infection.

Some agents such as interferon (INF) and lamivudine have been proved to be effective for chronic hepatitis B, but their efficacy is limited to a small percentage of highly selected patients[6-13]. The management of chronic hepatitis B remains a clinical challenge.

Traditional Chinese medicine (TCM) has a long history in treating hepatitis, and has been proven to have good curative effects and fewer side effects in treating acute and chronic liver diseases. HJT is a recipe for chronic hepatitis B, which can improve liver function and immunity of chronic hepatitis B patients as the seroconversion rate of HBeAg[14]. In order to analyze the immunoregulatory mechanisms of HJT, we treated chronic hepatitis B patients with HJT from June 1999 to March 2003 and

observed the clinical effect of HJT on T lymphocyte subset level, NK cell activity as well as TNF-α, IL-8, and sIL-2R level.

MATERIALS AND METHODS

Patients

A total of 85 patients with chronic hepatitis B were enrolled in this study and randomly divided into two groups. There were 27 males and 23 females aged 18-60 years (mean 36.9±9.5 years) in group I. There were 19 males and 16 females aged 18-60 years (mean 38.5±9.1 years) in group II. The difference in clinical data between the two groups was insignificant. Twenty age-matched healthy donors from the Blood Center of our hospital were assigned as group III. This prospective study was approved by the local ethics committee and written consent was obtained from the participants.

Diagnostic criteria

Patients with a history of hepatitis B or HBsAg carriers for at least 6 mo, who still had symptoms and signs of hepatitis as well as abnormal liver function and positive HBsAg, HBeAg and HBV-DNA, were diagnosed as chronic hepatitis B in the present study.

Criteria for enrollment

Patients, aged 18-60 years with their serum alanine aminotransferase (ALT) level being 80-240 μ/L and who had positive serum HBeAg and HBV-DNA, were enrolled. The diagnosis of hepatitis B was made in accordance with the standards for chronic viral hepatitis issued in the Fifth National Conference on Infectious Diseases and Parasitosis (Beijing, China, 1995).

Criteria for exclusion

Patients aged over 60 years or less than 18 years, patients in pregnancy or in breast feeding period; patients who had hepatitis C or other hepatic viral infection, autoimmune hepatitis and drug-induced hepatitis or alcoholic hepatitis; patients with severe complications of the cardiovascular, renal or hematopoietic system and patients with mental diseases, were excluded.

Group I was treated with HJT that consisted of 10 g Radix Bupleuri, 12 g Radix Scutellariae, 9 g Rhizoma Pinelliae, 30 g Radix Codonopsis Pilosulae, 6 g Radix Glycyrrhizae Praeparata, 9 g Fructus Ziziphi Jujubae, 30 g Rhizoma Polygoni Cuspidati, 8 g Radix Morindae Officinalis, 30 g Herba Hedyotis Diffusae. One dose was taken per day for 6 mo. Group II was treated with oxymatrine (200 mg, t.i.d.), compound vitamin B (2 tablets, t.i.d.), vitamin C (100 mg, t.i.d.), vitamin E (50 mg, t.i.d.), and ester capsule (2 tablets, t.i.d.) for 6 mo.

Patients who had normal serum ALT and sero-conversion of HBeAg and HBV DNA (quantitative PCR) after treatment were defined as responders while those with negative results as non-responders.

Recording and observation of symptoms and signs

The symptoms and signs of patients were recorded in detail using the "Clinical Observation Table" once a month before and during the treatment.

Etiological markers of hepatitis B

HBV-M and anti-HAV, anti-HCV, anti-HDV, and anti-EBV marks were detected by enzyme-linked immunosorbent assay (ELISA). HBV-DNA was detected by quantitative polymerized chain reaction (PCR).

Liver function

The patients had liver function examination every month during the treatment, including contents of serum proteins, total bilirubin (TB) and activities of ALT and AST (aspartate aminotransferase).

T-lymphocyte subsets and NK cell activity

T-lymphocyte subsets were detected by the single clone antibody APAAP method, NK cell activity was assayed by MTT colorimetry.

Detection of cytokines

The levels of TNF-α, sIL-2R, and IL-8 were detected by double antibody sandwich ELISA.

Statistical analysis

All statistical analyses were performed by χ^2 test and Wilcoxon rank sum test using SPSS software. $P<0.05$ was considered statistically significant.

RESULTS

Standard for efficacy evaluation

The clinical efficacy of treatment was evaluated according to the following standards. Markedly effective: chief symptoms including right upper abdomen pain, poor appetite, and abdominal distention disappeared; HBeAg and HBV-DNA turned negative; serum levels of ALT, AST, and TBIL restored to normal. Effective: chief symptoms were alleviated or improved; the level of HBV-DNA decreased; HBeAg did not turn negative; serum levels of ALT, AST, and TBIL decreased by >50% of the original levels. Ineffective: the chief symptoms or the serum levels of ALT, AST, and TBIL or HBeAg and HBV-DNA did not show any improvement.

Clinical efficacy of treatment

In group I, treatment was markedly effective in 7 cases, effective in 41 and ineffective in 2, the total effective rate being 96.0%. In group II, treatment was markedly effective in 0 cases, effective in 30, and ineffective in 5, the total effective rate being 85.7%. The difference in total effective rate was insignificant between the two groups ($P>0.05$) and the markedly effective rate was significantly different between the two groups ($P<0.05$).

Levels of ALT, AST, TB, and HBV-DNA before and after the treatment

After 6 mo of treatment, the levels of ALT, AST, and TB in two groups were obviously decreased ($P<0.01$). HBV-DNA level in group I was obviously decreased ($P<0.05$). HBV-DNA and HBeAg turned negative in seven patients and HBeAg turned negative in two patients but HBV-DNA did not turn negative. HBeAg turned negative in two patients of group II but HBV-DNA did not turn negative (Table 1).

T lymphocyte subsets before and after the treatment

The level of CD_4^+ cells was lower whereas the level of CD_8^+ cells (groups I and II) was higher in patients than in normal persons (group III) ($P<0.01$). There was no significant difference between the levels of CD_3^+ cells in patients and normal persons ($P>0.05$). After 6 mo of treatment, the level of CD_4^+ cells increased, whereas the level of CD_8^+ cells decreased ($P<0.05$) in group I. However, the levels of CD_4^+ and CD_8^+ cells had no significant difference in group II ($P>0.05$, Table 2).

Serum levels of TNF-α, sIL-2R, and IL-8 as well as NK activity before and after the treatment

The NK cell activity was lower whereas the levels of TNF-α, sIL-2R, and IL-8 was higher in patients (groups I and II) than in normal persons (group III) ($P<0.01$). After 6 mo of treatment, NK cell activity was significantly increased, whereas the levels of TNF-α, sIL-2R, and IL-8 decreased ($P<0.05$) in group I. However, there was no significant difference in group II ($P>0.05$, Table 3).

T lymphocyte subsets and NK activity of responders and non-responders of group I before and after the treatment

The levels of CD_3^+, CD_4^+, and CD_8^+ cells and NK cell activity in the two groups had no significant difference before treatment ($P>0.05$). After 6 mo of treatment, the level of CD_4^+ cells and NK cell activity increased, whereas the level of CD_8^+ cells decreased in responders ($P<0.05$). NK cell activity and the level of CD_4^+ and CD_8^+ cells in the non-responders had no significant difference after treatment ($P>0.05$, Table 4).

DISCUSSION

Though the pathogenesis of chronic hepatitis B remains unclear, a great many studies have shown that chronic hepatitis B patients are usually accompanied with disorder of immune function and hepatocyte damage is mainly caused by immunological injury[15–19]. Alterations of T

Table 1 Levels of ALT, AST, TB, and HBV-DNA before and after the treatment (mean±SD)

		n	ALT (U/L)	AST (U/L)	TB (μmol/L)	HBV-DNA (copy/mL)
Group III		20	21.52±8.90	15.56±7.65	11.75±5.71	<1 000
Group I	Pre-T	50	232.52±12.25	139.65±9.62	43.35±5.86	$(1.62\pm0.81)\times10^{8.31}$
	Post-T	50	33.26±9.35[b]	35.18±8.26[b]	19.95±5.12[b]	$(9.25\pm1.90)\times10^{5.02a}$
Group II	Pre-T	35	225.70±11.61	135.45±9.21	41.45±5.85	$(1.47\pm0.65)\times10^{8.22}$
	Post-T	35	30.86±8.95[b]	65.68±8.82[b]	29.55±546[b]	$(8.26\pm2.20)\times10^{7.62}$

Pre-T: before treatment; Post-T: after treatment; [a]$P<0.05$ *vs* before treatment in the same group; [b]$P<0.01$ *vs* before treatment in the same group.

Table 2 T lymphocyte subsets before and after the treatment (mean±SD)

		n	CD_3(%)	CD_4(%)	CD_8(%)	CD_4/CD_8
Group III		20	68.10±9.25	39.27±8.70	30.96±6.82	1.70±0.72
Group I	Pre-T	50	65.55±8.22	35.06±5.38[b]	34.80±4.36[b]	1.10±0.35[b]
	Post-T	50	67.35±8.85	37.60±8.52[a]	31.95±5.61[a]	1.31±0.42[a]
Group II	Pre-T	35	65.86±9.21	35.15±6.01[b]	35.10±6.56[b]	1.07±0.46[b]
	Post-T	35	66.71±9.56	35.92±8.55	34.66±6.25	1.12±0.36

Pre-T: before treatment; Post-T: after treatment; [b]$P<0.01$ *vs* group III ; [a]$P<0.05$ *vs* before treatment in the same group.

Table 3 Serum levels of TNF-α, sIL-2R, and IL-8 as well as NK activity before and after the treatment (mean±SD)

		n	TNF-a (mg/L)	sIL-2R (kU/L)	IL-8 (μg/L)	NK (%)
Group III		20	0.58±0.23	310.0±30.7	0.72±0.2	59.65±7.5
Group I	Pre-T	50	18.8±8.9[b]	390.9±12.0[b]	2.42±0.8[b]	43.12±6.5[b]
	Post-T	50	10.5±6.8[a]	310.22±8.9[a]	1.12±0.5[a]	52.90±7.0[a]
Group II	Pre-T	35	19.0±7.2[b]	395.7±16.5[b]	2.45±0.8[b]	43.02±6.8[b]
	Post-T	35	15.62±7.9	355.6±9.5	1.80±0.7	46.54±6.9

αPre-T: before treatment; Post-T: after treatment; [a]$P<0.05$ *vs* before treatment in the same group; [b]$P<0.01$ *vs* group III.

Table 4 T lymphocyte subsets in responders and non-responders of group I before and after the treatment (mean±SD)

		n	CD_3 (%)	CD_4 (%)	CD_8 (%)	NK (%)
Responders	Pre-T	7	66.02±8.86	35.10±4.76[b]	34.92±4.36[b]	43.52±7.1[b]
	Post-T	7	67.80±9.11	38.85±8.85[a]	30.15±5.82[a]	55.60±8.2[a]
Non-responders	Pre-T	43	65.50±9.08	34.92±6.30[b]	34.77±6.56[b]	42.93±6.7[b]
	Post-T	43	66.09±9.35	35.99±8.70	34.25±5.52	45.60±6.5
Group III		20	68.10±9.25	39.27±8.70	30.96±6.82	59.65±7.5

[a]$P<0.05$ *vs* before treatment in the same group; [b]$P<0.01$ *vs* group III.

lymphocyte subsets and NK cells are important reasons for the disorder of immune function due to HBV infection, TNF-α, IL-8, and sIL-2R are important cytokines associated with liver damage. Therefore, the importance of T lymphocytes and NK cells as well as cytokines in the occurrence of chronic HBV infection has received more and more attention.

CD_3^+, CD_4^+, and CD_8^+ cells are major function subgroups of T cells. An antiviral cellular immune response of CD_4^+ and CD_8^+ is the important mechanism of hepatocyte injury induced by HBV, the specific response of CD_4^+ and CD_8^+ to the virus antigen is closely related with the elimination of the virus[6,20,21]. NK cells play a critical role in host innate defense against viruses and are partly responsible for liver injury in the process of erasing viruses[22-28]. Recent studies found that NK cells are potent activators of dendritic cells (DCs), which have an impact on the magnitude and direction of DC activation of T cells under the conditions of chronic viral infection, activated NK cells can release cytokines and prevent virus from reproducing[23,29]. Therefore, T-lymphocyte subsets and NK activity can be considered as an appropriate response of immune system to inhibit viral replication and HBV eradication. In the present study, we discovered that in the outbreak period of chronic hepatitis B, NK activity and level of CD_4^+ cells were lower, whereas the level of CD_8^+ cells was higher in patients than in normal persons, suggesting that disorders of cellular immune function and pathologic damages occur in chronic hepatitis B patients.

The serum NK activity and CD_4^+ cell level in non-responders were lower than those in normal persons, whereas the level of CD_8^+ cells in non-responders was higher than that of normal persons. After treatment, the NK activity and CD_4^+ cell level were increased in seven patients with the conversion of HBV-DNA and HBeAg and the liver function resumed to normal. The results suggest that T-lymphocyte subsets and NK activity are depressed rather than activated in viral hepatitis B, but levels of T lymphocyte subsets and NK activity are closely related with different courses of hepatitis B. At the same time, levels of T lymphocyte subsets and NK activity in some patients were still low in palliative period, indicating that the chance of recrudescence might increase. T lymphocyte subsets and NK cells play a critical role in response to HBV infection and their level and mutual relation can be used to identify the cellular immune level in patients with chronic hepatitis B[11,38].

TNF-α plays an indispensable role in liver injury mediated by specific immune response to HBV infection[30]. Pretreatment with anti-TNF-α mAb in animal model strongly blocks Th1 cell-induced hepatocyte necrosis and apoptosis[32]. However, it was reported that TNF-α exerts its antiviral effects without destruction of hepatocytes[33]. IL-8 is a chemotactic factor of neutrophils and T cells and plays a role in hepatic injury in patients with chronic viral hepatitis. Remarkable increase of IL-8 leads to accumulation of cytotoxic T lymphocytes, which get direct and immediate access to the target hepatocytes and the resident intrahepatic macrophages, subsequently causing the damage of hepatocytes[34-36]. Release of sIL-2R from activated T lymphocytes may occur as a result of proteolysis of mIL-2R or as a result of alternative mRNA process. High level of sIL-2R in chronic HBV infection appears directly related to the activity of liver diseases; therefore, serum sIL-2R levels can be used to indicate the degree of liver damage in patients with chronic HBV infection[31,37,38].

In the present study, we discovered that in the outbreak period of chronic hepatitis B, the levels of IL-8, TNF-α, and sIL-2R were higher in patients than in normal persons during and after HJT treatment, significantly increased suggesting that cytokines and immunocytes may play a role in the pathogenesis of chronic hepatitis B.

HJT is a recipe for treating hepatitis in which cold and warm drugs are used to eliminate evils and restore healthy energy. Former research indicates that HJT can protect the liver from injury[20,21]. We discovered that HJT could improve liver function and NK activity, regulate T cellular immune function in chronic hepatitis B patients. The results suggest that HJT exerts its effect by improving the cellular immune function and decreasing inflammatory cytokines in chronic hepatitis B patients.

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• *BRIEF REPORTS* •

Herpes simplex virus type 1 in peptic ulcer disease: An inverse association with *Helicobacter pylori*

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Abstract

AIM: To assess the frequency of herpes simplex virus type I in upper gastrointestinal tract ulcers and normal mucosa with the modern and better assays and also with a larger number of well characterized patients and controls and its relationship to *Helicobacter pylori*(H *pylori)*.

METHODS: Biopsy specimens from 90 patients (34 with gastric ulcer of the prepyloric area and 56 with duodenal ulcer) were evaluated. Biopsies from 50 patients with endoscopically healthy mucosa were considered as the control group. The method used to identify herpes simplex virus-1 (HSV-1) was polymerase chain reaction. *H pylori* was detected by the CLO-test and by histological method.

RESULTS: Herpes simplex virus-1 was detected in 28 of 90 patients with peptic ulcer (31%) [11 of 34 patients with gastric ulcer (32.4%) and 17 of 56 with duodenal ulcer (30.4%)] exclusively close to the ulcerous lesion. All control group samples were negative for HSV-1. The likelihood of *H pylori* negativity among peptic ulcer patients was significantly higher in HSV-1 positive cases than in HSV-1 negative cases (P = 0.009). Gastric ulcer patients with HSV-1 positivity were strongly associated with an increased possibility of *Helicobacter pylori* negativity compared to duodenal ulcer patients (P = 0.010).

CONCLUSION: HSV-1 is frequent in upper gastrointestinal tract ulcers but not in normal gastric and duodenal mucosa. There is an inverse association between HSV-1 and *H pylori* infection.

Key words: HSV-1; Herpes simplex virus type 1; Peptic ulcer; Duodenal ulcer; Gastric ulcer; PCR; Polymerase chain reaction; *H pylori*; Non-steroidal anti-inflammatory drugs

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INTRODUCTION

During the last two decades, a significant progress has been made in the role of *Helicobacter pylori*(*H pylori*) in the pathogenesis of peptic ulcers, while the invention of new powerful antisecretory drugs has changed dramatically the treatment of the disease. However, the exact etiopathogenesis of peptic ulcer disease is still under investigation.

The significant role of gastric acidity and inflammation of mucosa due to *H pylori* cannot be disputed, but a multifactorial etiology for peptic ulcer disease seems to be emerging[1-3].

The idea of a possible correlation between HSV-1 and peptic ulcers has appeared almost 40 years before[4,5], due to many common characteristics observed in the clinical picture and the natural history of both diseases[4-7].

A possible involvement of HSV-1 in peptic ulcer disease was reported from several investigators, but a firm conclusion has not yet been reached. The vast majority of these studies are based on the detection of antibodies against the virus in the serum and the duodenal juice of patients with peptic ulcer[8-12], a finding also common in the apparent healthy population. There are only two studies that report the presence of HSV-1 in tissue samples obtained from gastric and duodenal ulcers, using polymerase chain reaction (PCR) methods, but the number of the examined populations is small[13,14]. On the other hand, in the studies reported above, a possible correlation between HSV-1 and *H pylori* has been investigated in the

pathogenesis of peptic ulcer disease.

DNA of HSV-1 has been detected also in human vagal[15] and celiac ganglia[16], which provide the nerve network to gastric tissue. Theoretically, since vagotomy is used to treat peptic ulcer disease, the same treatment may interrupt the migration of activated HSV-1 from ganglia to gastric mucosa, thus preventing the recurrence of ulcer.

The purpose of this study was to investigate the possible relationship between HSV-1 and peptic ulcer and whether viral infection of ulcer patients is related to the presence of *H pylori* infection.

MATERIALS AND METHODS

Patients and biopsies

All patients who underwent esophagogastroduodenoscopy at our institution from September 1999 to September 2002 were recruited. The first group included 56 patients (31 men and 25 women) with active duodenal ulcer (average 53.5±15 years, range from 19 to 83 years). The second group included 34 patients (22 men and 12 women) with active ulcer of the prepyloric area of the stomach (mean of 61.5±16.2 years, range from 22 to 89 years) and the third group that formed the control group, consisted of 50 patients (28 men and 22 women) with no evidence of pathologic findings (mean of 54.8±16.7 years, range from 21 to 86 years).

Tissue samples were taken from all 90 patients with peptic ulcer for the detection of HSV-1 in duplicate, from the following areas: the base and the rim of the ulcer; the adjacent area of the ulcer at a distance of 3 cm (minimal and maximal distance from the crater 3 and 5 cm respectively). For this reason, in the duodenal ulcer group a second duodenal biopsy was obtained; an endoscopically normal area of the stomach (the corpus in gastric ulcer cases and the antrum in duodenal ulcer cases).

Two samples were also taken from endoscopically healthy areas of the antrum and corpus of the stomach in all 50 controls.

Two samples from the antrum and two from the corpus of the stomach were taken for the detection of *H pylori* using the rapid urease test (CLO-test) and routine histology.

Finally, specimens from the gastric ulcers were examined histopathologically to exclude malignancy.

Risk factors probably involved in the pathogenesis of peptic ulcer, such as non-steroidal anti-inflammatory drugs (NSAIDs), smoking, alcohol, history of herpes labialis, family history of peptic ulcer, and ulcer site, were recorded and analyzed.

DNA extraction

Genomic DNA was extracted using the QIAamp DNA mini kit, following the protocol supplied for purification from fresh tissues. DNA was finally dissolved in 50–100 μL of TE buffer depending on the size of DNA pellets and stored at -20 °C until amplification.

Primer design

For the nested PCR assay, oligonucleotides deduced from the published sequence of the RL2 gene-coding region from HSV-1 were used[17]. For the control DNA assay, oligonucleotides for b-actin gene were used. The primer sequences and characteristics are shown in Table 1.

Table 1 Characteristics and nucleotide base sequences of primers used for nested PCR and control assays

Gene target	GenBank accession number	Product size (bp)	Sequences[1]	T_m (°C)
HSV-1: RL2	X14112	450	Outer sense agcagcgactctgaggcggagaccg	69.1
			Outer antisense tgcgggtctgggggtcgttcacga	71.4
		110	Inner sense cccggcagttgcgggggcgc	73.4
			Inner antisense aaggtgtcgcagcggcaggtg	60.8
b-actin		200	Forward gtgatctccttctgcatcc	53.2
			Reverse ctcttccagccttccttc	52.7

T_m, melting temperature. [1]Sequences shown are in the 5′ to 3′ direction.

Nested PCR amplification and detection assay of HSV-1

B-actin PCR generating a 200-bp product was performed to determine the DNA integrity of the samples. For the quality control PCR assay, the following program was used 1 cycle at 94 °C for 2 min; 35 cycles at 94 °C for 30 s, at 58 °C for 30 s, at 72 °C for 30 s; and a final cycle at 72 °C for 7 min.

PCR detection of HSV-1 was carried out in a 50 μL reaction mixture containing 25 μL of Taq PCR master mix solution (Qiagen), 13 μL of double-distilled DNase-free water, 1 μmol/L concentration of each primer and 10 μL of the extracted sample. PCR was performed on a PE 9 600 thermocycler (Perkin-Elmer Cetus, Branchburd, NJ, USA). The cycling conditions were at 94 °C for 1 min, 5 cycles at 94 °C for 5 s and at 72 °C for 4 min; 5 cycles at 94 °C for 5 s and at 70 °C for 4 min; 30 cycles at 94 °C for 5 s and at 68 °C for 4 min. After the final cycle, tubes were incubated for an additional 10 min at 72 °C. Nested PCR amplification was done with a 0.5 μL aliquot from the first run, 25 μL of Taq PCR master mix solution, 18 μL of double-distilled DNase-free water and 1 μmol/L concentration of each inner primer under the following cycling conditions: at 94 °C for 2 min; 35 cycles at 90 °C for 30 s, at 68 °C for 30 s, at 72 °C for 30 s and a final extension at

72 °C for 10 min. Each amplification run contained one negative and one positive control. The negative control consisted of blank reagent and water. For the positive control, HSV-1 genomic DNA provided by Sigma was used. Consistent PCR analyses were repeated twice or more.

The PCR products were analyzed by 2% agarose gel electrophoresis in 0.5×Tris–borate EDTA buffer along with ethidium bromide. A molecular weight marker (Φ× 174/Hae III, Sigma) was also run simultaneously to identify the molecular size of the PCR products. The DNA bands were visualized by UV transillumination and analyzed using a gel-documentation system. None of the PU and the control samples were negative in the b-actin test ultimately leaving 90 PU and 50 controls that were subjected to HSV-1 PCR analysis.

Helicobacter pylori testing

For the detection of *H pylori*, a CLO-test was used with high sensitivity and specificity (Kimberly-Clark CLO test, Ballard Medical products, Draper, UT 84020, USA)[18,19].

An experienced pathologist also assessed the histological sections with Giemsa stain[20].

Statistical analysis

All associations between parameters of interest were examined either by Fisher's exact test or Pearson's *chi square* test with continuity correction.

Multivariate analysis was performed using the stepwise logistic regression model to assess the contribution of the common risk factors to peptic ulcer development and *H pylori* detection.

$P<0.05$ was considered statistically significant.

RESULTS

Polymerase chain reaction

The genome of HSV-1 was present in 28 of the 90 patients with peptic ulcer (31.1%), contrary to the control group in which no positive detections were found (0%, $P<0.0005$, Figures 1-3). There was an equal prevalence in the two subgroups of patients, 17 of 56 patients with duodenal ulcer (30.4%) and 11 of 34 with gastric ulcer (32.4%) were tested positive ($P = 0.843$). In all HSV-1 positive cases, the viral genome was detected from the tissue samples obtained from the crater of the ulcer as from the samples obtained from the rim, while all samples from adjacent and distant areas were negative.

CLO test

All the *H pylori* positive subjects by CLO test from both groups were also positive for the bacteria with histology.

The incidence of *H pylori* was significantly higher in peptic ulcer patients (76/90, 84.4%) than in controls (30/50, 60%) ($P = 0.002$). A statistically significant difference was also found between patients with duodenal ulcer and those with gastric ulcer ($P = 0.036$). Negative *H pylori* was more frequently observed in patients with gastric ulcer (26.5%) than in patients with duodenal ulcer (8.9%, Table 2).

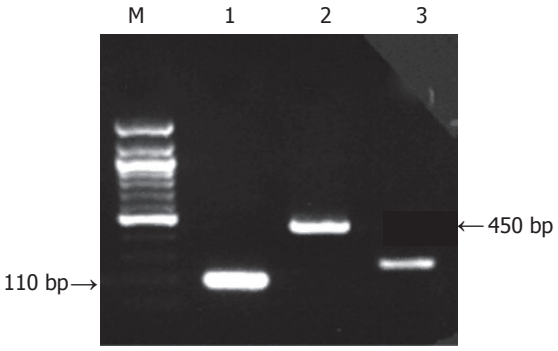

Figure 1 Nested PCR with HSV-1 outer and inner primers (450- and 110-bp amplicons: arrows) on 2% agarose gel, by ethidium bromide staining. Lane M: DNA molecular weight marker Φ×174/*Hae* III, lane 1: HSV-1 positive control (second run of nested PCR), lane 2: HSV-1 positive control (first run of nested PCR), lane 3: b-actin quality control PCR peptic ulcer sample.

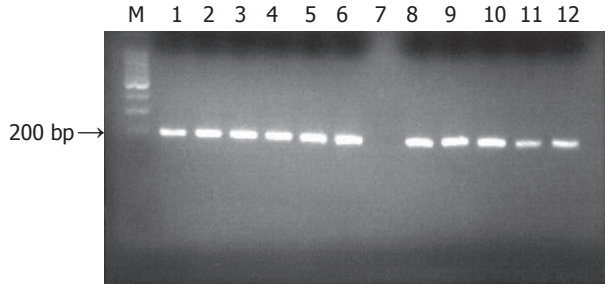

Figure 2 PCR quality assay control. Lane M: DNA molecular weight marker Φ ×174/Hae III, lanes 1-6: positive b-actin peptic ulcer samples; lane 7: negative control (without template); lanes 8-12: positive b-actin peptic ulcer samples.

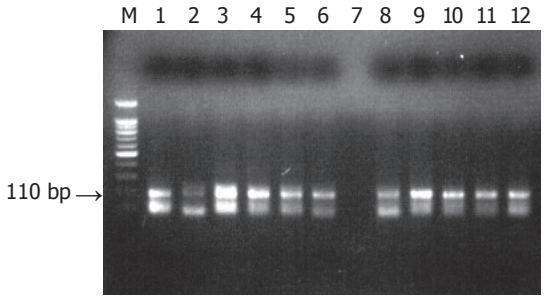

Figure 3 Nested PCR amplification of HSV-1 in samples of patients with peptic ulcer. Lane M: DNA molecular weight marker Φ×174/Hae III, lane 1: HSV-1 positive control (HSV-1 genomic DNA by Sigma), lanes 2-6: positive samples from patients with peptic ulcer, lane 7: negative control (without template), lanes 8-11: positive samples from patients with peptic ulcer, lane 12: HSV-1 positive control.

PCR and CLO test were significantly associated with respect to *H pylori* detection in all 90 peptic ulcer patients. Negative *H pylori* was more frequently detected in positive PCR samples (32.1%) than in negative PCR samples (8.1%) ($P = 0.009$) (Table 2). In the group of duodenal ulcer patients, *H pylori* negativity was more frequently observed in positive PCR samples (11.8%), than in negative PCR samples (7.7%). However, this difference was not

Table 2 Association between *H pylori* and HSV-1 in patients with peptic ulcer

	H pylori (+), %	*H pylori* (–)	*P*	Odds ratio (95%CI)
Peptic ulcer	76/90 (84.4)	14/90		
Controls	30/50 (60)	20/50	0.002	3.7 (1.6–8.1)
Gastric ulcer	25/34 (73.5)	9/34		
Duodenal ulcer	51/56 (91.1)	5/56	0.036	3.67 (1.1–12.11)
HSV-1 (+)	19/28 (67.9)	9/28		
HSV-1 (–)	57/62 (91.9)	5/62	0.009	5.4 (1.61–18.11)
HSV-1 (+)/GU	4/11 (36.4)	7/11		
HSV-1 (–)/ GU	21/23 (91.3)	2/23	0.002	18.37 (2.75–122.94)
HSV-1 (+)/ Duodenal ulcer	15/17 (88.2)	2/17		
HSV-1(–)/ Duodenal ulcer	36/39 (92.3)	3/39	0.634	-

HSV-1 (+)/gastric ulcer, patients with gastric ulcer positive for HSV-1; HSV-1 (–)/gastric ulcer, patients with gastric ulcer negative for HSV-1; HSV-1 (+)/duodenal ulcer, patients with duodenal ulcer positive for HSV-1; HSV-1 (–)/duodenal ulcer, patients with duodenal ulcer negative for HSV-1; CI, confidence interval.

Table 3 Association between *H pylori* and HSV-1 in relation with the site of peptic ulcer

	H pylori (+), %	*H pylori* (-)	*P*-value	Odds ratio (95% CI)
HSV-1(+)/ gastric ulcer	4/11 (36.4)	5/11	0.010	13.13 (1.92–89.5)
HSV-1(–)/ duodenal ulcer	15/17 (88.2)	2/17		

HSV-1 (+), patients positive for HSV-1; CI, confidence interval.

statistically significant ($P = 0.634$) (Table 2) whereas it was statistically significant in the subgroup of gastric ulcer patients ($P = 0.002$). In the gastric ulcer subgroup, *H pylori,* negative cases were observed in 63.6% of positive PCR samples and in 8.7% of negative PCR samples (Table 2). Finally, the likelihood of negative *H pylori* in HSV-1 positive samples in the group of gastric ulcer patients was significantly higher than that in the group of duodenal ulcer patients ($P = 0.010$) (Table 3).

Statistical analysis of other parameters

We also studied some of the common risk factors for the development of peptic ulcer disease. No statistically significant difference was found between patients and controls regarding family history of upper gastrointestinal ulcer, history of *Herpes labialis*, alcohol consumption, and use of NSAIDs (Table 4).

As expected, tobacco smoking was the only statistically significant risk factor for the development of peptic ulcers between patients and control population ($P = 0.019$). Smokers were associated with a 2.57-fold increase risk of peptic ulcer development.

The performed multivariate analysis also confirmed that *H pylori* and tobacco smoking (OR: 3.320 and 2.619 respectively) were more likely to induce peptic ulcer (Table 5).

Table 4 Risk factors and peptic ulcer disease

	Peptic ulcer patients (%)	Controls (%)	Odds ratio	*P*
History of *H labialis*				
Yes	24/90 (26.7)	9/50 (18.0)	-	0.302
No	66/90 (73.3)	41/50 (82.0)		
Alcohol consumption				
Yes	32/90 (35.6)	14/50 (28.0)	-	0.469
No	58/90 (64.4)	36/50 (72.0)		
NSAID use				
Yes	23/90 (25.6)	8/50 (16.0)	-	0.275
No	67/90 (74.4)	42/50 (84.0)		
Smoking				
Yes	45/90 (50.0)	14/50 (28.0)	2–57	0.019
No	45/90 (50.0)	36/50 (72.0)		
Family history of peptic ulcer				
Yes	30/90 (33.3)	9/50 (18.0)	2.3	0.076
No	60/90 (66.7)	41/50 (82.0)		

Table 5 Contribution of other risk factors to peptic ulcer development

	B	S.E	df	Sig.	Odds ratio	95%CI for Odds ratio
Smoking	0.963	0.394	1	0.015	2.619	1.209–5.673
H pylori infection	1.2	0.473	1	0.011	3.32	1.313–8.389
Age	0.011	0.014	1	0.432		
Sex	0.144	0.442	1	0.745		
History of *H labialis*	0.721	0.484	1	0.137		
Alcohol	0.021	0.492	1	0.966		
NSAID use	0.286	0.494	1	0.562		
Family history	0.44	0.48	1	0.359		
Constant	–0.741	0.393	1	0.06		

CI, confidence interval.

DISCUSSION

Despite the progress during the last 20 years in the understanding of the pathogenesis of peptic ulcer disease, it is clear that gastroduodenal ulcer is the result of a multifactorial process.

H pylori infection and NSAIDs have been recognized as the two most important causes of peptic ulcer disease. The proportion of peptic ulcers not associated with *H pylori* infection or the use of NSAIDs is increasing. Yet several studies have shown that 4.1-44% of peptic ulcers are not related to either of the two factors[21-25].

The possible involvement of HSV-1 in the process is a field of interest for several investigators, but a firm conclusion has not been reached. The presence of viral DNA in tissue samples obtained from the ulcer site is 9.5-18%[13,14]. The possible explanation for this finding is that the HSV-1 expression is prompted either by the ulcer injury or by immune cells[14,16] or HSV-1 itself might cause the ulcerative lesion by directly infecting the mucosal cells[13] or finally that HSV-1 expression is induced by the ulcer treatment[26-28].

In the present study, in a substantially larger number of patients than in the studies reported above (90 *vs* 22 and 21 respectively), the positivity for HSV-1 DNA was expressed using PCR in a greater percentage of samples (31% *vs* 9.5% and 18% respectively).

It should be noted that viral DNA was detected only in the tissue samples obtained from the base and the rim of the lesions, whereas all the other examined samples obtained from the adjacent ulcer areas and endoscopically healthy mucosa from the patients and the control group were negative for the viral genome.

This finding can be explained as follows. The HSV-1 may have initially entered the vagus ganglia through the oral pharynx or other peripheral connecting sites. Upon activation, the virus would travel down the vagal nerve to the potential site of peptic ulcer lesion. HSV-1 itself might cause ulcerative lesion in selected cases of a subset of peptic ulcer diseases by directly infecting the mucosal cells in the stomach and duodenum following virus release from neuroendocrine cells or vagal nerve terminals or both. Alternatively, peptic ulcer might activate latent HSV-1 in vagal ganglia, making replication of HSV-1, a contributing but not an initiating factor of ulcer[13].

The detection percentage of positive HSV-1 was similar between the group of patients with gastric and the group of patients with duodenal ulcer (32.4% and 30.4% respectively), in contrast to previous studies, where the viral DNA is demonstrated only in tissue samples from gastric ulcers[13,14]. The exact cellular localization of HSV-1 DNA could not be identified.

On the other hand, investigating a possible association between *H pylori* and HSV-1 in pathogenesis of a subset of gastroduodenal ulcers, our data suggest that the PCR HSV-1 positivity is associated with a 5.4-fold increase in negative *H pylori* detection. Moreover, the patients with ulcer lesions infected with HSV-1 presented a similar prevalence of *H pylori* infection as the control group, which was significantly lower than that in the HSV-1 negative ulcer cases ($P = 0.09$). This finding requires further investigation.

Possible interpretations for the increased HSV-1 DNA positive detection rate in *H pylori* negative ulcers include the following. *H pylori* negativity is influenced by the viral expression, HSV-1 negativity influences *H pylori* infection and the virus independently causes some gastroduodenal ulcers.

According to our results, in the subgroup of patients with duodenal ulcers, the risk of *H pylori* infection was independent from HSV-1 DNA expression ($P = 0.634$). On the other hand, in the subgroup of patients with gastric ulcer disease, the possibility of *H pylori* negativity was 18.5-fold higher ($P = 0.002$). These data are in accordance with those reported by Lohr *et al*.[13].

Additionally, according to our results, there was not any correlation between peptic ulcer disease and age, sex, ulcer site, family history of gastroduodenal ulcers, history of *H. labialis*, alcohol consumption and NSAIDs use. On the contrary, statistically significant difference ($P = 0.019$) was observed between patients with peptic ulcers and controls, as far as smoking was concerned.

Our results indicate an involvement of HSV-1 in the pathogenesis of peptic ulcer disease. Although an opportunistic infection with the virus in the ulcer site cannot be excluded, the inverse relationship between HSV-1 detection and *H pylori* infection indicates a possible implication of this virus in the formation of the ulcer crater, at least in a subgroup of patients. Furthermore, experimental data support this[13,15,16,30,31]. The exact localization of the virus in ulcer tissue cells should be precisely determined in order to clarify whether the lesion is caused by HSV-1 or the virus opportunistically is established, especially in immunocompromised patients[32].

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• *BRIEF REPORTS* •

Endoscopic mucosal resection for high-grade dysplasia and intramucosal carcinoma in Barrett's esophagus: An Italian experience

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Abstract

AIM: To evaluate endoscopic mucosal resection (EMR) in patients with high-grade dysplasia (HGD) and/or intramucosal cancer (IMC) in Barrett's esophagus (BE).

METHODS: Between June 2000 and December 2003, 39 consecutive patients with HGD (35) and/or IMC (4) underwent EMR. BE >30 mm was present in 27 patients. In three patients with short segment BE (25.0%), HGD was detected in a normal appearing BE. Lesions had a mean diameter of 14.8±10.3 mm. Mucosal resection was carried out using the cap method.

RESULTS: The average size of resections was 19.7±9.4×14.6±8.2 mm. Histopathologic assessment post-resection revealed 5 low-grade dysplasia (LGD) (12.8%), 27 HGD (69.2%), 2 IMC (5.1%), and 5 SMC (-12.8%). EMR changed the pre-treatment diagnosis in 10 patients (25.6%). Three patients with SMC underwent surgery. Histology of the surgical specimen revealed 1 T0N0 and 2 T1N0 lesions. The remaining two patients were cancer free at 32.5 and 45.6 mo, respectively. A metachronous lesion was detected after 25 mo in one patient with HGD. Intra-procedural bleeding, controlled at endoscopy, occurred in four patients (10.3%). After a median follow-up of 34.9 mo, all patients remained in remission.

CONCLUSION: In the medium term, EMR is effective and safe to treat HGD and/or IMC within BE and is a valuable staging method. It could become an alternative to surgery.

Key words: Endoscopic mucosal resection; Barrett's esophagus; High-grade dysplasia; Intramucosal cancer

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INTRODUCTION

The incidence of adenocarcinoma (AC) of the esophagus has increased in the last three decades in the Western world[1-3]. Most esophageal adenocarcinomas arise in a precursor lesion, Barrett's esophagus (BE). The esophageal cancer risk in BE patients is about 1 cancer per 200 patient-years, or 0.5% per year[4,5]. The prognosis is poor for the typical patient who presents with invasive cancer, with a 5-year survival rate of under 10%[6].

Dysplasia arising in BE is a marker of progression toward invasive cancer. High-grade dysplasia (HGD) is an uncommon but a serious problem. In 16-60% of patients found to have HGD, invasive cancer was diagnosed in the next 5-7 years[6,7], although spontaneous regression of HGD can also occur[8]. Following surgical esophagectomy for HGD, 10-50% of cases had previously undetected foci of invasive cancer found in the resected specimen[9,10].

When HGD is detected, the three options that are available are: endoscopic surveillance, esophagectomy, and endotherapy.

Surveillance in patients with HGD is controversial. The uncertainty of natural history of HGD and its slow progression rate, could justify a contemplative attitude. Schnell *et al.* evaluated the long-term outcome of 75 patients with HGD who were enrolled in an endoscopic

surveillance program. After a mean follow-up of 7 years, AC occurred in 12 (16%) patients[7].

Esophagectomy has been the standard treatment of HGD and early cancer in BE. However esophagectomy is associated with surgical morbidity of 20-50% and mortality of about 3%, even at high-volume centers. In patients older than 70 years, mortality was 11%[11,12]. A less invasive treatment would be desirable. A newer alternative to esophagectomy is endoscopic mucosal resection (EMR). Improved diagnosis of early malignancy in BE, including endoscopic ultrasound (EUS), may change the therapeutic approach. The superficial lesions of HGD and intramucosal cancer (IMC), with minimal risk of lymph node metastasis, can be removed by EMR. This procedure allows adequate histologic assessment and definitive treatment.

The use of EMR to treat HGD and IMC in BE is increasing, but the number of published series remains less. Our aim was to evaluate EMR in the treatment of HGD and IMC in Barrett's patients in terms of complications and recurrence rate.

PATIENTS AND METHODS

Patients

Between June 2000 and December 2003, 39 consecutive patients (mean age 62.8±11.4 years) with histologically confirmed HGD (35) or IMC (4) in BE underwent EMR in three Departments of Gastroenterology acting as regional referral centers. All patients were previously identified by endoscopic examination performed in our centers or referred from other hospitals. In this case, the original histologic slides were re-evaluated by two expert pathologists (G.L., V.V.) on BE before EMR.

This study was approved by our institutional review board. All patients gave written informed consent to endoscopic therapy. Patients were evaluated and treated using the same protocol by one of the authors representing each of the three institutions.

At endoscopy, superficial lesions were defined using the following classification: slightly elevated (0-IIa), flat (0-IIb), and slightly depressed (0-IIc)[13]. Endoscopy was performed with standard diagnostic videoendoscopes (GIF-Q145, Olympus Optical Co. Ltd., Tokyo, Japan). Patients with ulcerated lesions seen on endoscopy were excluded.

Before EMR, EUS with mechanical rotating transducer, using the water-filling method (GF-UMQ130, 7.5-20 MHz, Olympus Optical Co. Ltd.), was routinely performed to assess lesion depth and mediastinal lymph node status. All patients had a CT scan of the thorax. Only lesions confined to the mucosal layer with no apparent lymph node metastases were considered for EMR.

Deep sedation with propofol was used. EMR was performed using a plastic cap (MH-594, Olympus Optical Co. Ltd, Tokyo, Japan) preloaded on the tip of a standard diagnostic forward-viewing endoscope. The cap had an outer diameter of 13 mm and a length of 15 mm. Inside the distal end of the cap was a gutter, which positions the opened polypectomy snare. A 2-mm segment of the gutter was removed with a scalpel before placement on the endoscope tip. This modification was then aligned with the operative channel to avoid interference by the injection needle or other devices (hemoclips, biopsy forceps). Submucosal injection of epinephrine solution (1:60.000-1:100.000) to create a fluid cushion was performed using variceal injection needles (VIN-23, Wilson Cook Medical Inc; Variject Contrast Injection Needle, Boston Scientific) in all patients. Injected volume ranged between 8 and 30 mL, depending on the lesion diameter. Methylene blue was added to the solution for visual enhancement of the fluid cushion in contrast to the lesion. The cap was next applied against the lesion, which was aspirated into it. A monofilament polypectomy snare (SD-221U-25, Olympus Optical Co. Ltd, Tokyo, Japan) was then firmly secured around the tissue and resection was performed. Resection was performed by endocut mode only, using the ERBE-ICC 200 cautery device (ERBE Elektromedizin GmbH, Tubingen, Germany). The output setting predefined by the manufacturer was adopted: cut 120 W, coagulation 60 W. To minimize interobserver variability among the three endoscopists performing EMR, the diameter of the lesion and the resected areas were estimated by placing an open polypectomy snare around the lesion. In patients with lesions ⩽12 mm wide "en-bloc" resection was performed. For larger lesions, piecemeal resection was completed by applying the cap close to the previously resected area. Specimens were aspirated into the cap and all materials were retrieved for histopathologic assessment. To complete EMR, multiple withdrawals and re-intubations were needed. No overtube was used.

Intra-procedural bleeding (during the EMR) was controlled by epinephrine-saline injections (1:10.000) and, when required, by placing hemoclips (HX-600-090L; rotatable clip fixing device HX-6UR-1, Olympus Optical Co., Ltd).

All patients were hospitalized for 48 h. They were kept fasting for 24 h, then a soft diet was advised for the next two weeks. After EMR, an intravenous PPI (omeprazole, 40 mg/d) was administered for 24 h, followed by a maintenance oral dose of 40 mg/d.

Surveillance

Following EMR, patients were contacted by phone weekly in the first fifteen days, then monthly, to monitor for symptoms such as dysphagia. Endoscopy was repeated at 3, 6, and 12 mo and then yearly, with multiple biopsies from the EMR site, and four-quadrant biopsies from the residual BE.

Complete remission was defined, when well demarcated areas of squamous re-epithelialization without mucosal irregularities, were observed. A lesion was considered metachronous, when diagnosed more than 12 mo from the EMR, irrespective of its location.

Histology

Following the WHO guidelines, we defined HGD by cytologic and architectural changes confined to the

mucosa. IMC was defined by cytologic and architectural changes confined to the lamina propria. Invasive cancers were considered to be those infiltrating the submucosa (SMC)[14,15].

Statistical analysis

Statistical data were expressed as mean±SD. The Kruskal-Wallis test was used to compare histologic severity with lesion size. A P value of 0.05 or less was considered statistically significant.

RESULTS

EMR was performed in 39 patients, 34 males and 5 females. Their mean age was 62.8±11.4 years. Thirty-six patients had type 0-IIa mucosal abnormalities and three had HGD detected by random biopsies in a normal appearing BE.

Mean Barrett's length was 4.3±2.5 cm. Long segment BE (LSBE, ⩾3 cm) was present in 27 patients. The three patients with non-visible lesions had short segment BE (SSBE). These patients underwent EMR with the aim of completely removing the metaplastic epithelium.

Lesions had a mean diameter of 14.8±10.3 mm. Histologic severity did not correlate with lesion size. The average size of reconstructed resected specimens was 19.7±9.4×14.6±8.2 mm. In all patients the EMR was completed in one session. "En-bloc" resection was performed in 19 cases with lesions of ⩽12 mm. The size of the first resected specimen by EMR-C method ranged between 8 and 12 mm.

Following EMR, the pre-treatment histology was re-classified in 10/39 patients (25.6%). Among the 35 initially diagnosed as HGD, five were found to have only LGD, but three had SMC. Of the four patients initially diagnosed as IMC, two were re-classified as SMC. EUS did not help to identify submucosal infiltration.

Table 1 shows the characteristics of patients and lesions according to the histology found in the EMR resection specimens.

Table 1 Characteristics of patients and lesions according to histology of mucosa resected at EMR (mean±SD)

	LGD	HGD	IMC	SMC (no surgery)
Age (yr)	60.6±8.8	61.76±11.8	56±4.2	73.6±8.3
BE length (cm)	6.6±4.1	3.6±1.8	3.7±1.8	5.8±3.1
Size of lesion (mm)	22.5±8.7	13±10.2	16±5.7	17.6±11.9
Metachronous lesion (%)				
No	5 (100)	24 (88.9)	2 (100)	2 (100)
Yes	-	1 (3.7)	-	-
No follow-up endoscopy (%)	-	2 (7.4)	-	-
Total	5	27	2	2

LGD: low-grade dysplasia; HGD: high-grade dysplasia; IMC: intramucosal cancer; SMC: submucosal cancer; BE: Barrett's esophagus.

Cancers

The five patients with SMC had a mean age of 73.6± 8.3 years. The mean BE length was 5.8±3.1 cm, and lesion size ranged from 5 to 30 mm. The lesions were type IIa (superficial elevated). Histologic assessment detected tiny areas of low-grade differentiation, and in two of them, lymphatic permeation. Three (7.7% of all patients) underwent esophagectomy and the histopathologic assessment showed one T0N0 and two T1N0. The remaining two patients were considered unfit for surgery due to advanced age (81 and 84 years) and/or comorbidities (cardiovascular disease). They were included in the surveillance program.

Complications

Intra-procedural bleeding occurred in four patients (10.3%), and was controlled with epinephrine injections in two, and with epinephrine plus clipping in the other two. Delayed bleeding was not seen. No patient needed blood transfusion. No perforations occurred. Retrosternal pain was present in one patient. An esophageal stenosis developed 8 mo later in a patient with LSBE (7 cm). He had a 30 mm HGD lesion and the diameter of the EMR area was 40 mm. He was successfully treated by a single bougienage.

Surveillance

Follow-up endoscopy was performed in 32 of the 34 patients without invasive cancer (94.1%), two patients declining repeat examination. The follow-up ranged from 16.3 to 72.1 mo (median 34.9 mo). In one patient (3.1%), with an original lesion of 20 mm (HGD), a metachronous lesion was detected after 25 mo. It was easily removed by EMR, and the histology showed HGD. One of the two patients with SMC, who did not undergo surgery died of cardiovascular disease 45.6 mo later, and the other was alive and cancer free at a 32.5 mo surveillance.

EUS was repeated at 3 and 6 mo after EMR. CT of the thorax and upper abdomen were also performed after 6 mo, and then after 1 year to evaluate the lymph node status and the presence of metastases. Figures 1A and 1B show endoscopic appearance of a SSBE with HGD before and three months after EMR.

DISCUSSION

The use of EMR for EC in the digestive tract was described by Inoue *et al*.[16]. We employed EMR to remove 39 focal esophageal HGD and IMC lesions of 3-30 mm size in BE, with few complications. Most lesions were flat mucosal abnormalities. Systematic biopsies were taken from the remainder of the BE to exclude non-visible multifocal lesions.

The data reported in the present study represent the first experience of EMR in BE, in Italy. Three major referral centers joined in the effort of evaluating the clinical outcome of EMR. In a period of three years a relatively small number of patients has been included in our study, especially when compared with the series

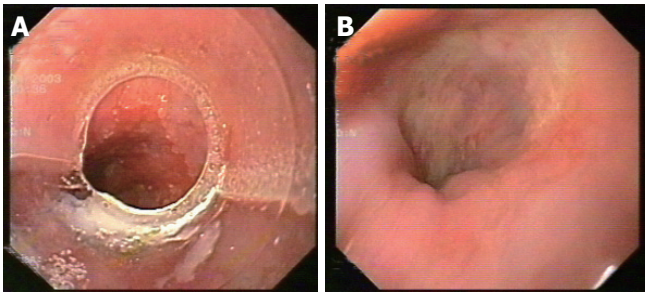

Figure 1 Endoscopic appearance of SSBE with HGD before (**A**) and 3 mo after EMR (**B**).

reported by German authors[17]. This difference is attributable to the still limited number of patients with early neoplastic lesions in BE, detected in endoscopic centers. Furthermore, as surgery is still the gold standard treatment for HGD and IM, the majority of these patients is referred for esophagectomy.

EMR could become a management option for HGD, and also for IMC, where the risk of lymph node involvement is from 0% to 4%[18,19]. Unfortunately, in cases with SMC, the incidence of regional lymph-node metastasis is 15-50%[20-22]. Current data support surgical resection in the setting of submucosal infiltration by AC, unless comorbidity or advanced patient age precluded it.

Table 2 displays data from selected studies on EMR in BE since 2000. Most published studies report EMR of endoscopically visible areas of HGD. Some of these studies differ in methodology and it is difficult to compare them. However, our results are in accordance with the data reported by other authors. EMR provides greater diagnostic precision than endoscopic biopsy, despite endoscopy with biopsies and standard EUS before EMR in all patients. In five of 39 cases, undetected SMC was found on histological examination of the resected specimen. Reclassification of the histology after EMR occurred in 26% of our patients. Other authors have reported reclassification in 0% to 75% of cases after EMR (Table 2). Causes may include biopsy sampling error and observer interpretation variability.

Bleeding is the most frequent adverse event with EMR, reported in a median 10% of patients. Intra-procedural bleeding also occurred in 10% of our patients and was managed endoscopically without transfusion. Esophageal stenosis is a late complication of EMR, reported in 0-30% of cases (Table 3). In our study, one patient (2.5%) developed stenosis. Larger EMR resections may increase the risk; in a study of 137 patients, stenosis was seen only when EMR involved more than two-thirds of the esophageal circumference[23]. However, in one report of circumferential EMR, only two of 12 patients developed stenosis[24]. The perforation risk is generally less than 1%. No perforations occurred in our series. Overall, complications seem fewer for EMR than for surgical resection. In one study, complications occurred in 48% of esophagectomies *vs* 16% for EMR combined with photodynamic therapy (PDT)[25].

A recent controlled study of 100 mucosectomies compared the cap method and a ligation method for suction EMR. The diameter of the removed specimen, the diameter of the resected area, and the complication rate showed no significant differences between the two groups, and no severe complications occurred[26].

There is limited information on the long-term effectiveness of EMR. May *et al.* followed 70 patients with HGD or early AC for a mean of 34 mo after EMR. Ten percent had minor complications. During follow-up, 21/70 patients were found to have locally recurrent or metachronous disease, treated endoscopically with success in all but one case. The only death from Barrett's AC was in a patient who had surgery for SMC[17]. In our series, follow-up for a median 35 mo was available in 94.1% of patients. One of the 39 patients (2.6%) had a metachronous lesion after 25 mo, successfully treated with another EMR. According to other authors, malignant transformation of HGD is about 34% in 6-54 mo[27], corresponding to our follow-up period. In this time range we did not find invasive AC. In our two patients with SMC who did not undergo surgery, no histologic evidence of disease was detected.

Table 2 Selected studies on EMR in Barrett's esophagus

Author	Number of patiens	Size of lesions cm (mean)	Technique	Histology pre-EMR	Histology post-EMR	Change in diagnosis (%)	Complications	Follow-up months (mean)	Recurrence
Seewald *et al*. 2003 Germany[24]	12	Median 5	EUS Snare	5 HGD/ IMC (visible) 7 IMC (non-visible)	2 BE 1 LGD 5 HGD AC 4	75%	Bl: 33% Stricture:17%	Median: 9	0
Ahmad *et al*. 2002 USA[33]	19/101	0.5-3	EUS EMR-C Snare injection	AC 6 HGD 6 NOS 7	AC 8 HGD 4 LGD 1 Benign 6	58%	Bl: 11%	>=24	0
May *et al*. 2002 Germany[17]	80	Nos	EUS EMR±PDT	HGD 7 EC 73	AC: 11/80	Nos	Bl: 6% Stricture: 4%	34	24/78

Table 3 Selected studies on EMR in Barrett's esophagus

Author	Number of pts	Size of lesions cm (mean)	Technique	Histology pre-EMR	Histology post-EMR	Change in diagnosis (%)	Complications	Follow-up Months (mean)	Recurrence
Buttar *et al*. 2001 USA[34]	17	8	EUS VLD-PDT Injection	IMC: 7 AC: 10	IMC: 7 AC: 10	47%	Bl: 6% Stricture: 30%	13	HGD (1)[1] AC (1)
Nijhawan *et al*. 2000 USA[35]	25	7	EUS Lift-and-cut VLD Injection	2 BE 8 LGD 5 HGD 9 AC 1 other	2 BE 3 LGD 5 HGD 13 AC 2 other	48%	0	14.6	0
Ell *et al*. 2000 Germany[36]	35	0.9	EUS EMR± injection	HGD: 3 EC: 32	HGD: 3 EC: 32	0	Bl: 20%	12	11%

Pts: patients; EMR: endoscopic mucosal resection; EUS: endoscopic ultrasonography; HGD: high-grade dysplasia; IMC: intramucosal carcinoma; BE: Barrett's esophagus; LGD: low-grade dysplasia; AC: invasive adenocarcinoma; Bl: bleeding; EMR-C: EMR with cap; PDT: photodynamic therapy; Nos: not otherwise specified; VLD: variceal ligator device; EC: early cancer; [1]persistence of HGD.

We used EMR to remove focal lesions, but did not attempt to resect long circumferential segments of Barrett mucosa. This might seem a logical extension of the use of EMR. Removing both focal lesions and also the remainder of the BE might give greater assurance that no neoplasia or columnar mucosa remained than with PDT or thermal methods, as well as providing complete histological assessment of the mucosa. Potential risks and technical difficulties have so far limited the use of circumferential EMR, but this has now been tried. Experimentally, we assessed the feasibility of 3 cm circumferential EMR in a porcine model using EMRC. One out of four pigs developed a severe stenosis[28]. This work was advanced by Rajan *et al*. who performed more extensive EMR without complications[29]. In the clinical setting, Satodate *et al*. resected an entire 5 cm circumferential BE, together with 2 cm of gastric mucosa. The patient had early multifocal AC, with one small area of submucosal invasion. EMR was performed using the cap method in a single session, 30 separate pieces of the mucosa being removed. Following dilatations for esophageal stenosis, at 10 mo he was asymptomatic and endoscopy showed no stenosis, no recurrent cancer, and no remaining BE[30]. Seewald *et al*. performed circumferential EMR in 12 patients with HGD and IMC. Seven had no visible lesions. A monofilament polypectomy snare without a cap was used. In each case, the entire BE (median length 5 cm) was completely removed in 1-5 sessions, with a median number of 5 snare resections per endoscopic session. Four patients had minor bleeding, and two required esophageal dilations[24].

Recent advances in techniques as chromoendoscopy with methylene-blue and high-magnification endoscopy may help in identifying non-visible dysplastic lesions and in recognizing their width in Barrett's esophagus. Chromoendoscopy with methylene-blue may be useful to detect dysplastic mucosal areas. In fact, about 90% of these areas are unstained[31]. High-magnification endoscopy allows the identification of specific pit-patterns of the esophageal epithelium. Dysplasia seems to distort this pattern[32]. In our three patients with non-visible lesion in SSBE, we did not use these techniques because we performed EMR with the aim to completely remove the metaplastic epithelium.

Our study confirms that EMR is a feasible, low risk procedure to treat focal HGD and IMC within BE. Given encouraging short and medium-term results, endoscopic therapy is more often being considered as primary treatment[17]. However, controlled studies comparing EMR and esophagectomy are not available. Further experience is needed to determine the place of total removal of Barrett' s mucosa by a more extensive EMR.

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• *BRIEF REPORTS* •

Effect of cytokine gene polymorphism on histological activity index, viral load and response to treatment in patients with chronic hepatitis C genotype 3

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Abstract

AIM: To investigate the association between cytokine gene polymorphism and disease status in chronic hepatitis C genotype 3 by liver biopsy, ALT, HCV RNA levels and response to treatment.

METHODS: Patients with chronic hepatitis C genotype 3 were analyzed for single nucleotide polymorphisms of interleukin (IL)-10, IL-1 beta, interferon-gamma (IFN-γ), tumor necrosis factor-alpha (TNF-α) and transforming growth factor-beta (TGF-β) by polymerase chain reaction using sequence-specific oligonucleotide primers. Liver biopsies were assessed by modified histological activity index (HAI) scoring system using a scale of 0–18 for grading the necro-inflammatory activity and 0–6 for staging the fibrosis. HCV RNA levels were determined by bDNA assay. The patients were treated with interferon alpha and ribavirin for 6 mo. Sustained virological response was assessed 6 mo after the completion of the treatment.

RESULTS: Out of the 40 patients analyzed, 26 were males. Mean age was 40.5±12.5 years (range 18–65 years). The frequencies of different dimorphic polymorphisms based on single nucleotide substitution were as follows: IL-10-1082 G/A 85%, A/A 12.5%, G/G 2.5%; IL-10-819 A/C 87.5%, C/C 10%, A/A 2.5%; IL-10-592 C/A 72.5%, C/C 27.5%; IL-1 C 90%, U 10%; IFN-874 T/A 50%, T/T 27.5%, A/A 22.5%; TNF-308 A/G 95%, G/G 5%; TGF-10 T/C 52.5%, C/C 35%, T/T 12.5%. The mean grades of necro-inflammatory activity of different genotypes of IL-10 at promoter site -1082 were A/A = 3.6, A/G = 5.0, and G/G = 10.0 and the difference was significant (P = 0.029). The difference in the stage of disease at a scale of 0–6 was A/A 0.8, A/G 2.3, and G/G 4.0 (P = 0.079). The difference in the HAI seemed to be related to the presence of allele -1082G. For IL-10 -819 genotypes, mean scores of fibrosis were A/A = 6.0, A/C = 2.2, and C/C = 1.0 (P = 0.020) though the inflammatory activity was not much different. No significant differences in HAI were noted among polymorphisms of other cytokines. Moreover, ALT and HCV RNA levels were not significantly different among different cytokine polymorphisms. There was a significant correlation of HAI and HCV RNA levels with the duration of disease. TGFβ -10 genotype CC patients had a better end of treatment response than those with other genotypes (P = 0.020). Sustained virological response to the treatment was not influenced by the cytokine polymorphism. No effect of other factors like viral load, degree of fibrosis, gender, steatosis, was observed on sustained virological response in this population infected with genotype 3.

CONCLUSION: There is no significant correlation between cytokine polymorphisms and HAI except for the polymorphisms of anti-inflammatory cytokine IL-10, which may influence hepatic inflammatory activity and fibrosis in patients with chronic hepatitis C genotype 3. Sustained virological response in this genotype does not seem to be influenced by cytokine gene polymorphisms.

Key words: Interleukin; Interferon gamma; Tumor necrosis factor alpha; Transforming growth factor; Cytokines; Gene polymorphism; Hepatitis C; Alanine aminotransferase; Liver biopsy

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INTRODUCTION

HCV infection is a leading cause of chronic liver disease worldwide. The infection leads to viral persistence and chronic disease in a very high proportion of cases. Pathogenesis of liver injury is not fully understood. There is a complex relationship between HCV and its host. Liver lesions could be the result of immune

responses or cytopathic action of the virus. Cytotoxic T cells and cytokines produced by both CD4+ (T helper) and cytotoxic T cells may be responsible for much of the damage that occurs in the livers of infected patients[1].

Two distinct patterns of cytokine production may occur[2]. Type 1 responses are characterized by production of interleukin-2 (IL-2), tumor necrosis factor-alpha (TNF-α) and interferon-gamma (IFN-γ), which are prime and maintain antigen-specific cellular immunity[3,4] and are important in defense against viruses. Type 2 responses are characterized by IL-4, IL-5, and IL-10, which promote humoral immune responses. An imbalance in helper T-cell type 1 (Th1) and type 2 (Th2) cytokines is suggested to play an important role in the pathogenesis of chronic hepatitis C. The progressive liver injury seen in chronic HCV infection is associated with the upregulation of intrahepatic Th1-like cytokines. Intrahepatic IFN-γ and IL-2 mRNA expression is upregulated in chronic hepatitis C, while the expression of IL-10, a Th2-like cytokine, is downregulated[5] .

Intrahepatic CD4+ T cells play a pathogenetic role in the hepatic injury of HCV infection[6]. Vigorous HCV-specific CD4+ Th1 response, particularly against the nonstructural proteins of the virus, may be associated with viral clearance and protection from disease progression[7]. Patients without viremia after HCV infection frequently have strong Th lymphocyte responses of the Th1 type to multiple HCV antigens many years after the onset of infection, whereas antibody responses are less marked. These results suggest that control of HCV replication may depend on effective Th lymphocyte activation[8,9]. There is also an enhanced Th2 response during chronic HCV infection, which may partly be responsible for the persistence of HCV infection.

In addition to the altered intrahepatic cytokine expression, there might be a significant correlation between circulating cytokines and degree of inflammation in the liver. One study has shown such a correlation between baseline TNF levels and histologic grading score of hepatitis[10]. The maximal capacity of cytokine production varies between individuals and may correlate with polymorphism in cytokine gene promoters. The objectives of our study were to analyze the role of allelic or genotype variations of IL-10, IL-1 beta, IFN-γ, TNF-α and TGF-β and its association with hepatocellular injury as suggested by liver biopsy and ALT and treatment outcome. We selected genotype 3 for this study which is the main genotype in our country.

MATERIALS AND METHODS

Out of the 40 patients analyzed, 26 were males. Mean age was 40.5±12.5 years (range 18-65 years). The participants did not receive interferon therapy and had neither co-infection with human immunodeficiency virus and hepatitis B virus nor other associated forms of chronic liver disease.

Quantitative serum HCV RNA was determined by bDNA assay (Bayer, USA) according to the manufacturer's instructions. The minimum quantification limit of the assay was 3 000 HCV RNA copies/mL serum. HCV genotyping was performed using PCR and reverse hybridization assay (Innogenetics, Belgium). All patients included in this study were of HCV genotype 3. For cytokine gene polymorphism, DNA was extracted by proteinase K digestion from peripheral mononuclear cells, followed by phenol chloroform extraction and ethanol precipitation. After amplification polymorphisms in IL-10 (592, 819, 1082), IL-1β (-511), IFNγ (874), TNFα (308) and TGF (codon25) were examined as described previously[11-13]. The number in parenthesis indicates the location of the polymorphism on the DNA sequence. Briefly, single nucleotide polymorphisms (SNPs) were determined using sequence specific oligonucleotide primers. Each PCR reaction consisted of 1× PCR buffer, 0.2 mmol dNTPs. Concentration of $MgCl_2$ varied with the type of SNP examined, 50 ng of each polymorphism specific primer, 1 U of *Taq* polymerase in a final volume of 10 μL. To monitor PCR inhibition, growth hormone gene was simultaneously amplified as an internal control. Thermal cycling was performed in a Perkin-Elmer 9700 thermal cycler. Following PCR amplification, amplicons were stained with ethidium bromide and visualized on a UV transilluminator. The size of product generated in each PCR assay was ascertained and scored as positive/negative for the presence/absence of a particular polymorphism. The PCR product obtained with IL-1β-511 specific primers was digested with the restriction enzyme *Ava*II. The digested product was then visualized for the presence of restriction fragments.

Liver biopsy specimens were analyzed by a single pathologist, who was unaware of the patient's identity, treatment regimen, response, or timing of the biopsy relative to the treatment. Liver biopsies were assessed by modified histological activity index (HAI) scoring system[14] using a scale of 0-18 for grading and 0-6 for staging. Degree of steatosis was scored at a scale of 0-3. Presence or absence of lymph follicles was also documented.

Duration of disease was determined by calculating time interval from the exposure to a possible risk factor. Patients were treated with standard doses of interferon alpha (3 mega units subcutaneous, thrice a week) and ribavirin (800-1 200 mg/d) for 6 mo and followed up for another 6 mo. HCV RNA was repeated 6 mo after the treatment to document sustained response.

Statistical analysis

Statistical analysis was performed by two-tailed tests. *P* values were calculated by one-way analysis of variance (ANOVA), Pearson's χ^2 and Spearman's rho correlation tests. $P<0.05$ was considered statistically significant.

RESULTS

The frequencies of different dimorphic polymorphisms based on single nucleotide substitution were as follows:

IL10-1082 G/A 85%, A/A 12.5%, G/G 2.5%; IL10-819 A/C 87.5%, C/C 10%, A/A 2.5%; IL10-592 C/A 72.5%, C/C 27.5%; IL-1 C 90%, U 10%; IFN-874 T/A 50%, T/T 27.5%, A/A 22.5%; TNF-308 A/G 95%, G/G 5%; TGF-10 T/C 52.5%, C/C 35%, T/T 12.5%. The mean grades of necro-inflammatory activity at a scale of 0-18 for different genotypes of IL10 at promoter site -1082 were A/A = 3.6, A/G = 5.0, and G/G = 10.0 and the difference was significant (P = 0.029). The difference in the stage of disease at a scale of 0-6 was A/A 0.8, A/G 2.3, and G/G 4.0 (P = 0.079). This difference in the HAI seemed to be related to the presence of allele -1082G. For IL 10 -819 genotypes the mean scores of fibrosis were A/A = 6.0, A/C = 2.2, and C/C = 1.0 (P = 0.020) though the inflammatory activity was not much different.

No significant differences in the degree of necro-inflammatory activity and fibrosis were noted among the polymorphisms of other cytokines (Table 1). Moreover, ALT and HCV RNA levels were not significantly different among different cytokine polymorphisms. There was a significant correlation of duration of disease with grade and stage of disease and HCV RNA levels (P = 0.017, 0.018, and 0.015 respectively with Spearman's rho test).

Out of the 40 patients, 34 remained under the follow-up. These patients completed the 6-mo treatment with interferon and ribavirin. HCV RNA was repeated 6 mo after the treatment to document the sustained response and the effect of cytokine gene polymorphism. TGFβ -10 genotype CC patients had a better end of treatment response than those with other genotypes (P = 0.020), though there was no difference in the sustained virological response. No effect of other factors like viral load, degree of fibrosis, gender, steatosis, was observed on the sustained virological response in this population infected with genotype 3.

DISCUSSION

Approximately 80-90% of patients acutely infected with hepatitis C virus develop persistent infection, about one-half of them have elevated transaminases indicative of ongoing liver inflammation[15]. In the context of an inflammatory response against the virus, variable cytokine response of the host may be responsible for the variable liver damage. Moreover, the cause of viral persistence during HCV infection may be the development of a weak antiviral immune response to the viral antigens, with corresponding inability to eradicate infected cells or insensitivity of the virus to such cytokines or insufficient production of cytokines[16]. Thus, the continuing inflammation results in liver damage in the absence of complete virologic recovery[17].

Studies have shown that active liver injury in chronic hepatitis C patients is associated with increased circulating Th1 cytokine IL-2 but not with Th2 cytokine IL-10[18]. It has also been shown by some workers that serum alanine transaminase and the hepatic fibrosis levels are related directly to the frequencies of peripheral memory effector CD8(+) T cells producing IFN-γ (Tc1), but inversely to the frequencies of those producing both IL-4 and IL-10 (Tc2)[19]. Moreover, most liver-infiltrating T cells in chronic hepatitis C are type 1 cells. Studies to date in liver tissue showed that intrahepatic mRNA for type 1-like cytokines, such as IL-1β, IL-2, IL-6, IL-8, TNF-α, and IFN-γ were upregulated in chronic HCV infection[20]. The level of expression of type 1 cytokines, such as IL-2 and IFN-γ, is correlated with the degree of histologic injury as well as the likelihood of non-responsiveness to IFN-α therapy[21]. The presence of an ongoing cellular immune response probably also contributes to the process of hepatic fibrosis. Kupffer cells can be activated by the production of cytokines such as TNF-α, which in turn produce TGF-β[22].

Serum levels of different cytokines may not give the true picture of what is going on in the liver. For example, the mean IL2Rs and IFN serum levels are much higher in patients with anti-HCV than in the control group,

Table 1 Statistical significance of effects of cytokine gene polymorphisms on different parameters

Cytokine gene polymorphism	ALT (upper limit normal)	HCV RNA level	Grade of inflammation (0-18)	Stage of fibrosis (0-6)	Steatosis (0-3)	Lymph follicles	End of treatment response	Sustained response
IL 10 -1082 (GA, AA, GG)	0.636	0.241	0.029	0.079	0.267	0.848	0.078	0.331
IL 10 -819 (AC, CC, AA)	0.794	0.781	0.412	0.020	0.57	0.295	0.063	0.331
IL 10 -592 (CA, CC)	0.582	0.198	0.243	0.281	0.551	0.626	0.416	0.283
IFNγ -874 (AA, TT, TA)	0.389	0.848	0.919	0.921	0.955	0.382	0.21	0.933
TNFα -308 (AG, GG)	0.952	0.436	0.777	0.307	0.32	0.85	0.674	0.451
TGFβ -10 (CC, TC, TT)	0.734	0.72	0.386	0.959	0.232	0.643	0.026	0.206
IL 1 (C/U)	0.144	0.591	0.964	0.826	0.326	0.836	1.00	1.00

P values were calculated by ANOVA and Pearson' s χ^2 test. Statistically significant values. IL: interleukin; IFNγ: interferon gamma; TNFα: tumor necrosis factor alpha; TGFβ: transforming growth factor beta.

whereas the mean IL4 and IL6 levels are lower in patients infected with HCV[23]. Another study shows the higher levels of serum IL-1β, IL-4 and IL-6 (0.221, 0.104 and 1.393 pg/mL) in all HCV patients than in healthy adults (0.188, 0.025 and 0.600 pg/mL)[24].

Sustained response to interferon and ribavirin, defined as undetectable HCV RNA at 6 mo after discontinuation of therapy, is achievable in 30-60% of treated patients[25,26]. Predictors of response include viral factors such as viral genotypes, viral load and early disappearance of HCV RNA after initiation of therapy, while the host factors include gender, age and degree of fibrosis[25-27]. In recent years, increasing attention has been drawn to the role of host variation in cytokine levels in inflammatory and immune responses. Polymorphisms in genes encoding immunoregulatory proteins, proinflammatory cytokines, and fibrogenic factors may affect the production of these factors and influence disease progression in patients with chronic liver disease due to alcohol, primary biliary cirrhosis, or hepatitis C[28].

There might be an association of cytokine gene polymorphism and susceptibility to hepatitis C infection. Hohler *et al*.[29] have reported such an association with the polymorphism at the TNFα promoter. The TNF promoter variants TNF2 (-238A) and TNF3 (-308A) confer a 3.2-fold and 5.1-fold risk of cirrhosis respectively (P = 0.03 for both). Reciprocal effects have been observed with several TNF alleles and haplotypes defined by the -238 G/A and -308 G/A dimorphic sequences, thus polymorphisms in the TNF alpha promoter appear to be associated with the variability in the histological severity of chronic hepatitis C infection[30]. In our study done on hepatitis C genotype 3 patients, no such effect of TNFα -308 variability was observed.

Interleukin (IL)-10 is a cytokine that downregulates the proinflammatory response and has a modulatory effect on hepatic fibrogenesis and is a potent anti-inflammatory Th2 cytokine that downregulates the expression of major histocompatibility complex (MHC) class I and class II molecules, as well as the production of Th1 cytokines[31-36]. IL-10 levels differ widely between individuals, possibly because of polymorphisms in the promoter region of the IL-10 gene[37,38]. Specifically, three SNPs in the promoter (at positions -1082, -819, and -592 relative to the transcription start site) form three SNP combinations (ATA, ACC, GCC), which are associated with differential IL-10 expression[38-40]. It is reasonable to assume that hepatitis C patients who produce high levels of IL-10 have less hepatocellular injury and less ability to control infection and patients with low secretion of IL-10 have a better ability to eliminate the hepatitis infection. Perhaps low IL-10 production can skew the immune system into the Th1 type of response, facilitating the clearance of viral load. -1082A allele is associated with reduced IL-10 production *in vitro*[39]. In our study, individuals who were homozygous for IL-10 AA at position -1082 had a lower HAI.

It has been shown that hepatitis C patients, genotyped as high IL-10 producers, have a poor response to IFN-α therapy[40]. Such polymorphisms may also predict the sustained viral response to antiviral therapy[41]. These patients may benefit from additional treatment strategies designed to enhance T-helper type 1 (Th1) response. In one study, the interleukin-10 -1082 G/G genotype was identified more frequently in patients than in controls (P = 0.048). The patients exhibiting transforming growth factor-beta 1+29 (codon 10) C/C genotype variables were less likely to respond to treatment than patients with the T/T or T/C genotypes[42]. Liver transplant recipients, who are genotyped as having a low production profile of IL-10, are more prone to rejection and less likely to have hepatitis C recurrence[43]. The IL-1beta-31 genotype T/T or the IL-1beta-511/-31 haplotype C/T is associated with the presence of HCC in Japanese patients with chronic HCV infection[44].

However, not all the studies favor such effect of polymorphisms. Three members of the interleukin-1 gene family (IL-1A, IL-1B and IL-1RN), three polymorphic sites in the interleukin-10 gene promoter (-1082, -819, -592) and two in the TNF-α promoter (-308, -238) were studied in two independent DNA banks, each with appropriate controls. Standard PCR-based genotyping techniques were used. No significant difference in the distribution of any of the polymorphisms has been found in either study set[45].

We, in this study, determined SNP at position -1082, -819 and -592 in case of IL-10, -874 for IFN-γ, -308 for TNF-α, -10 for TGF-β and IL-1 C/ U and analyzed the frequency of their distribution and correlation with the ALT and HCV RNA levels, HAI and response to treatment. In our series, we selected genotype 3 patients because this is the main genotype in our region and not enough data are available on the influence of host cytokine gene polymorphisms of this viral genotype. Another reason of selecting a single genotype was to make the group uniform as different genotypes have different response rates to antiviral therapy and influence of cytokines may also be different. We could not find any significant difference in the cytokine genotype profile while analyzing different variables except for some influence of polymorphisms of IL-10 on liver histology. These polymorphisms did not modulate the response to interferon plus ribavirin therapy. This may be due to the small sample size or the fact that viral genotype 3 is easy to treat genotype in any case.

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• *BRIEF REPORTS* •

Gastroprotective activity of *Nigella sativa* L oil and its constituent, thymoquinone against acute alcohol-induced gastric mucosal injury in rats

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Abstract

AIM: To evaluate the role of reactive oxygen species in the pathogenesis of acute ethanol-induced gastric mucosal lesions and the effect of *Nigella sativa* L oil (NS) and its constituent thymoquinone (TQ) in an experimental model.

METHODS: Male Wistar albino rats were assigned into 4 groups. Control group was given physiologic saline orally (10 mL/kg body weight) as the vehicle (gavage); ethanol group was administrated 1 mL (per rat) absolute alcohol by gavage; the third and fourth groups were given NS (10 mL/kg body weight) and TQ (10 mg/kg body weight p.o) respectively 1 h prior to alcohol intake. One hour after ethanol administration, stomach tissues were excised for macroscopic examination and biochemical analysis.

RESULTS: NS and TQ could protect gastric mucosa against the injurious effect of absolute alcohol and promote ulcer healing as evidenced from the ulcer index (UI) values. NS prevented alcohol-induced increase in thiobarbituric acid-reactive substances (TBARS), an index of lipid peroxidation. NS also increased gastric glutathione content (GSH), enzymatic activities of gastric superoxide dismutase (SOD) and glutathione-S-transferase (GST). Likewise, TQ protected against the ulcerating effect of alcohol and mitigated most of the biochemical adverse effects induced by alcohol in gastric mucosa, but to a lesser extent than NS. Neither NS nor TQ affected catalase activity in gastric tissue.

CONCLUSION: Both NS and TQ, particularly NS can partly protect gastric mucosa from acute alcohol-induced mucosal injury, and these gastroprotective effects might be induced, at least partly by their radical scavenging activity.

Key words: *Nigella sativa*; Thymoquinone; Ulcer; Antioxidant; Rat

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INTRODUCTION

Intragastric application of absolute ethanol has long been used as a reproducible method to induce gastric lesions in experimental animals[1]. Gastric lesion is accompanied with the formation of free radicals (FRs) and reactive oxygen species (ROS)[2-4]. These radicals in particular seem to play an important role in ulcerative and erosive lesions of the gastrointestinal tract[5], as they attack and damage many biological molecules. Therefore, treatment with antioxidants and FR scavengers can decrease ethanol-induced gastric mucosal damage[6,7].

The black seed, *Nigella sativa* L (NS), a member of the family of ranunculaceae, contains more than 30% of fixed oil and 0.4-0.45 % wt/wt of volatile oil. The volatile oil contains 18.4-24% thymoquinone (TQ) and 46% many monoterpenes such as *p*-cymene and α-piene[8]. Recently, clinical and animal studies have shown that extract of the black seeds have many therapeutic effects such as immunomodilative[9], antibacterial[10], hypotensive[11], hepatoprotective[12] and antidiabetic effects[13]. Ohkawa *et al*.[14] also reported that NS oil and its derivative TQ inhibit eicosanoid generation in leukocytes and membrane lipid peroxidation. However, the gastroprotective effect of this plant and its major constituent against ethanol-induced gastric mucosal injury remains unclear.

In the present investigation, we studied the influence of NS on gastric mucosal lesions and the redox state induced by ethanol and to compare its actions with those of its constituent TQ.

MATERIALS AND METHODS

Plant materials and extraction procedure

The NS seeds were purchased from a local herb store, Van, Turkey. Voucher specimens were kept at the Department of Biochemistry, Yuzuncuyil University, Van, Turkey, for the future reference. The seeds of NS were powdered in a mixer, placed in a distillation flask and the volatile oil with 0.2% yield was collected by steam distillation. TQ 2-isopropyl-5-methyl-1,4-benzoquinone, was purchased from Sigma (St. Louis, MO, USA).

Treatment of rats

Forty male Wistar albino rats, weighing 200-250 g (aged 4 mo), were supplied by The Center of Medical Investigations of Yuzuncu Yil University. The animals were fed with a standard rat chaw (Murat Food Factory, Ankara, Turkey) and allowed to drink water *ad libitum*, but they were deprived of food 12 h before the experiment. The animals were housed in a single temperature controlled (20-25 °C) cage in dark/light cycle. All procedures were performed in sterilized conditions. All animals received human care according to the criteria outlined in the "Guide for the Care and Use of Laboratory Animals" prepared by the National Academy of Sciences and published by the National Institutes of Health.

Rats were assigned into 4 groups (each containing 10 animals). Control group was given physiologic saline orally (10 mL/kg body weight) as the vehicle (gavage), ethanol group was administrated 1 mL (per rat) absolute alcohol by gavage. The third and fourth groups were given NS (10 mL/kg body weight) and TQ (10 mg /kg body weight p.o) respectively 1 h prior to alcohol intake. One hour after ethanol administration, the animals were euthanized by cervical dislocation. The stomach was excised, cut along the greater curvature, and gently rinsed in tap water. The stomach was stretched on a piece of cork with mucosal surface up, and then examined in a standard position for macroscopic examination. Scoring of ulcer was performed with the help of magnifying glass. Lesion size (mm) was determined by measuring each lesion and its greatest diameter was recorded in the case of petechial lesions. Four such lesions were considered to be the equivalent of an 1 mm ulcer. The sum of the total severity scores in each group of rats divided by the number of animals, was expressed as the mean ulcer index (UI).

Biochemical analysis

Stomachs were cut into small pieces and homogenized in 0.15 mol/L ice-cold KCl using Heidolf Diax 900, type 595 (Germany) to give 20% homogenates. The homogenates were then made into aliquots and used for the assessment of antioxidant parameters.

MDA levels were determined as previously described[15]. Less than 0.2 mL of 10% (w/v) tissue homogenate, 0.2 mL of 8.1% sodium dodecyl sulfate (SDS), 1.5 mL of 20% acetic acid solution adjusted to pH 3.5 with NaOH, and 1.5 mL of 0.8% aqueous solution thiobarbituric acid (TBA) were added into the sample. The mixture was made up to 4.0 mL with distilled water, and heated at 95 ^{0}C for 60 min. After cooling, 1.0 mL of distilled water and 5.0 mL of the mixture of *n*-buthanol and pyridine (15:1, v/v) were added and shaken vigorously. After centrifugation at 4 000 r/min for 10 min, the organic layer was taken and its absorbance at 532 nm was measured. Total TBA-reactive materials were expressed as MDA, using a molar extinction coefficient for MDA of 1.56×10^5 cm/mol/L. MDA level was expressed as nmol/g.

Reduced glutathione (GSH) was determined according to the method described by Sun[16]. The GSH concentration (μmol/g) was computed from a standard curve constructed using different concentrations of standard GSH.

Stomach homogenate (20%) was centrifuged at 10 000 *g* for 10 min for 30 min at 4 ^{0}C (Beckman XL-70, USA). Following centrifugation, the supernatant (cytosolic fraction) was carefully removed from the pellet and used directly for assay of the enzymatic activities of SOD, GST and CAT.

SOD activity was detected according to Sun and Habig[17]. One SOD unit was defined as the enzyme amount causing 50% inhibition in the $NBTH_2$ reduction rate. SOD activity was also expressed as U/mg protein of stomach tissue sediment.

Gastric GST activity was determined according to the method of Clairborne *et al*.[18]. In brief, the GST activity toward 1-chloro-2,4-dinitrobenzene in the presence of glutathione as a co-substrate was measured specrophotometrically at 25 °C. The enzyme activity was determined by monitoring the changes in the absorbance at 340 nm for 4 min at 1-min intervals. The enzymatic activity was expressed as nmol min/g tissue.

CAT activity was determined according to the method of Lowry[19]. In short, the supernatant (50 μL) was added to a quartz cuvette containing 2.95 mL of 19 mmol/L H_2O_2 solution prepared in potassium phosphate buffer (0.1mol/L, pH 7.4). The change in absorbance was monitored at 240 nm over a 5-min period using a spectrophotometer (Shimadzu UV-1201, Japan). Commercially available CAT was used as the standard. CAT activity was expressed as U/g tissue.

The amount of protein was determined by the Lowry method[20].

Statistical analysis

The data were expressed as mean±SD and analyzed by repeated measures of variance (ANOVA). Tukey test was used to test for differences among means for which ANOVA indicated a significant ($P<0.05$) F ratio.

RESULTS

Oral administration of absolute ethanol produced multiple mucosal lesions in the rat stomach. Pretreatment with NS and TQ inhibited the ethanol-induced gastric mucosal injury in rats. Pretreatment with single oral dose of NS significantly reduced the ulcer index compared to alcohol

($P<0.01$). TQ also significantly inhibited ethanol-induced gastric lesions ($P<0.05$). Ulcer index (UI) is shown in Figure 1.

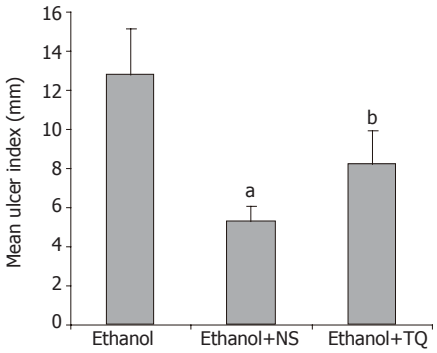

Figure 1 Mean UI in ethanol, ethanol + NS and ethanol + TQ treated rats. $^{b}P<0.01$, $^{a}P<0.05$ *vs* ethanol group

Administration of ethanol increased the MDA level in rat gastric tissue. In contrast, pretreatment with NS significantly decreased the MDA levels as compared to ethanol. TQ also significantly decreased the gastric MDA content, but to a lesser extent than NS. GSH activity decreased in the gastric tissue after ethanol administration, but pretreatment with NS or TQ increased the GSH activity gastric tissue compared to ethanol (Table 1). Prior administration of NS to rats markedly increased the gastric activity of SOD. Likewise, TQ increased the enzymatic activity of SOD compared to alcohol (Table 1). Pretreatment with either NS or TQ increased the enzyme activity of gastric GST. Neither NS nor TQ had any effect on the CAT activity of gastric mucosa (Table 1).

DISCUSSION

ROS are continuously produced during normal physiologic events, and removed by antioxidant defence mechanisim[21]. In pathological conditions, ROS are over produced and result in lipid peroxidation and oxidative damage. The imbalance between ROS and antioxidant defence mechanisms leads to oxidative modification in the cellular membrane or intracellular molecules[22]. Recent studies showed that ROS are one of the important factors in the pathogenesis of ethanol-induced mucosal damage[7,23,24]. Some ROS scavengers or inhibitors such as melatonin have protective effects on indomethacin- or ethanol-induced acute gastric injury in rats[25,26]. The cytoprotective role of antioxidants in the prevention and healing of gastric lesions has been widely investigated in a number of studies[24,27].

In the present study, administration of absolute alcohol by gastric gavage induced marked damage to the gastric mucosa that was obvious by macroscopic examination. The lesions were elongated hemorrhage and confined to the glandular portion with the highest subjective ulcer-scoring rate.

It was reported that alcohol causes severe oxidative stress in gastric tissue manifested as stimulated lipid peroxidation by increasing MDA content and decreasing of gastric GSH content[3,24,27].

The gastric activities of SOD and GST notably decreased following alcohol intake. The CAT activity, however, was unchanged. These results are in line with previous reports that demonstrated marked alterations in the enzymatic antioxidants following acute administration of alcohol to rats[3,28]. Depletion of non-protein sulfhydryl concentrations[1], modulation of nitric oxide sytem[29], reduction of mucosal blood flow[30], and autonomic nervous system regulation[31], are involved in the development of gastric lesions. One of the major mechanisms underlying the induction of gastric erosions by absolute alcohol is the oxidative damage with its dual events of lipid peroxidation and oxygen reactive species generation. Actually, oxygen-derived radicals have been implicated in the pathogenesis of gastric tissue damage and ulcerogenesis[6,32,33].

Pretreatment with a single oral dose of NS could partly reduce the ulcer index and promote healing of gastric lesions induced by acute intake of alcohol in rats. NS significantly decreased the gastric MDA content while it increased the gastric level of GSH compared to alcohol. The gastric activities of both SOD and GST were markedly elevated following administration of NS, whereas the CAT activity was not altered. Likewise, prior administration of TQ to animals could protect gastric mucosa and ameliorate most of the biochemical adverse effects induced by alcohol application, but to a lesser extent than NS.

These findings are in good agreement with a recent study by El-Denshary *et al.*[34]. The anti-ulcerogenic effects of NS can be attributed to the improvement of

Table 1 Effects of alcohol intake alone and following administration of either NS or TQ on the contents of MDA and GSH and activities of SOD, GSH and CAT in rat gastric tissue (means ± SD)

Parameters	Control	Ethanol	Eth + NS	Eth + TQ
MDA (nmol/g proteine)	136.33 ± 11.42	329.43 ± 19.80[a]	112 ± 17.16[c]	201.15 ± 29.11[c]
GSH (μmol/g proteine)	0.84 ± 0.04	0.44 ± 0.03[a]	0.54 ± 0.05[c]	0.42 ± 0.02
SOD (U/g proteine)	47.39 ± 5.74	17.75 ± 3.02[a]	56.88 ± 4.09[c]	51.08 ± 3.17[c]
GST (nmol/min/g proteine)	4.39 ± 0.29	1.81 ± 0.32[a]	4.02 ± 0.31[c]	4.07 ± 0.28[c]
CAT (U/g proteine)	56.45 ± 3.30	59.5 ± 7.99	55.63 ± 6.52	54.18 ± 10.19

$^{a}P<0.05$ *vs* control group. $^{c}P<0.05$ *vs* alcohol group.

the antioxidant status of animals due to an increase in mucin content of the gastric mucosa[34], or the presence of FR scavenging substances such as TQ[35]. It was also reported that NS given to sensitized quinea pigs, inhibits FR generation, and increases serum levels of SOD and glutathione[14]. NS could protect the gastric mucosa by increasing the bioavailability of arachidonic acid, resulting in biosynthesis of the cytoprotective prostaglandins in the stomach[36]. NS has also been reported to produce a marked inhibition on the release of leukotrienes, which cause mucosal tissue injury and hypoxemia[37]. Therefore, it may alter the delicate balance between prostaglandins and leukotrienes in the gastric mucosa favoring cytoprotection. TQ is the main active component of NS, and is able to inhibit lipid peroxidation[14]. Moreover, its ability to preserve the cell membrane integrity can be proven by the restoration.

In conclusion, pretreatment with NS and TQ, particularly NS can partly protect the gastric mucosa against the injurious effects of absolute ethanol and promote ulcer healing. NS or TQ can also mitigate most of the biochemical adverse effects induced by alcohol instillation in gastric tissue. Further studies are required to clarify the anti- ulcer and antioxidant action of NS or TQ.

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• *BRIEF REPORTS* •

Giardiasis in patients with dyspeptic symptoms

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Abstract

AIM: To investigate the prevalence of giardiasis in patients with dyspeptic symptoms.

METHODS: Clinical records of consecutive patients who attended Gastroenterology Department at Aga Khan University Hospital from January 2000 to June 2003 and had esophagogastroduodenoscopy (EGD) with duodenal biopsies and international classification of diseases 9th revision with clinical modifications (ICD-9-CM) coded with giardiasis were studied.

RESULTS: Two hundred and twenty patients fulfilled the above criteria. There were 44% (96/220) patients who were giardiasis positive, 72% (69/96) of them were males and 28% (27/96) of them were females. There were 65% (81/124) males and 35% (43/124) females who were giardiasis negative. The mean age of patients with giardiasis was 28±17 years, while that of giardiasis negative patients was 40±18 years (P<0.001). In patients with giardiasis, abdominal pain was present in 71% (68/96) of patients (P = 0.02) and diarrhea in 29% (28/96) (P = 0.005); duodenitis in 25% (24/96) on EGD (P = 0.006) and in 68% (65/96) on histopathology (P = 0.002).

CONCLUSION: Giardiasis occurs significantly in young people with abdominal pain, while endoscopic duodenitis is seen in only 25% of giardiasis positive cases, which supports routine duodenal biopsy.

Key words: Abdominal pain; Giardiasis; Stool examination; EGD; Duodenal biopsy

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INTRODUCTION

Giardia lamblia is the most common protozoan isolated from the gastrointestinal tract[1]. Worldwide incidence is believed to range from 20% to 60%[2]. The incidence rate is 2-7% in industrialized nations[3]. Patients with giardiasis typically present with diarrhea, vague abdominal discomfort, nausea and distention together with mild weight loss and lassitude. The absence of these symptoms may result in a low clinical index of suspicion for the diagnosis. Giardiasis is diagnosed by signs and symptoms, as well as the presence of giardia cysts and trophozoites in the stool. Stool examination can be unreliable, as organisms may be excreted at irregular intervals which can produce a false negative test result[4]. There is no gold standard for the diagnosis of giardiasis. The initial method of diagnosis is by demonstration of the trophozoite or cysts of *G lamblia* in the stool by microscopy or stool antigen detection by ELISA. Other methods of diagnosis include examination of duodenal contents by aspiration or biopsy with endoscopy. A definitive diagnosis may require repeated stool examinations, fecal immunoassays, or even sampling of the upper intestinal contents. Two stool examinations can detect 80-90% of infections, while three samples detect >90%[5]. About 20% of infestations are symptomatic, and do not continue for more than 3 mo along with the passage of cysts[2]. Difficulties are often encountered in finding the underlying cause of recurrent abdominal pain. Giardiasis is considered as an infrequent cause of dyspepsia[6], but this might not be true in a third world country. Prevalence of giardiasis is the highest in areas of poor sanitation and drinking water treatment. It is transmitted by eating and drinking contaminated food and water or fecal-oral contact. In immunocompetent patient, small bowel biopsy may show normal histology or villous architecture, but increased intraepithelial lymphocytes and plasma cells in the lamina propria or villous atrophy and inflammatory cells[7]. Giardia trophozoites can be found on the surface or penetrating the epithelium down to the lamina propria[7]. The aim of this study was to investigate the prevalence of giardiasis in patients with dyspeptic symptoms.

MATERIALS AND METHODS

Patients

We carried out a retrospective analysis of medical records of all the patients who attended endoscopy unit of gastrointestinal section at the Aga Khan University Hospital from January 2000 to June 2003 consecutively for dyspeptic symptoms. They had an esophagogastrodu odenoscopy (EGD) with duodenal biopsies after routine examination including stool examination. The giardiasis negative group consisted of the patients who had a stool examination and an EGD with duodenal biopsy and were not diagnosed as giardiasis. The giardiasis positive group consisted of the patients who had positive EGD with duodenal biopsy and stool examination for giardia cysts or trophozoites. Clinical symptoms at the time of presentation, diagnosis, drug treatment dosage and duration, past history of giardiasis, neutrophil and lymphocyte counts from complete blood picture, random blood glucose and stool examination were noted. Patients with diagnosis of celiac disease were excluded from the analysis.

Stool examination

Stool parasitological analysis of samples collected from spontaneous bowel movement was performed. Samples were collected in a sterile container and transported soon after to the laboratory for examination. Microscopically each stool specimen was examined in a fresh normal saline smear and Lugol's iodine preparation.

Esophagogastroduodenoscopy

All endoscopic examinations were performed by staff-members of our hospital's gastroenterology section, using an Olympus video scope GIF xQ 140. Duodenitis was diagnosed when scattered. Reddened raised non-eroded mucosal patches were endoscopically identified in the duodenal bulb and descending duodenum.

Histopathology

Hematoxylin and eosin (HE) staining of duodenal biopsy was used for pathological confirmation of giardia trophozoites. Most organisms were tangentially cut and many were seen as small sickle-shaped objects near the epithelial surface.

Statistical analysis

The SPSS (Release 11.5.0, standard version, copyright © SPSS; 1989-02) was used for data analysis. Descriptive analysis was done for demographic and clinical features. Results were presented as mean±SD for continuous variables, and number (percentage) for categorical variables. Univariate analysis was performed by using the independent sample *t*-test for continuous variables and Pearson's chi-square test for categorical variables to assess the demographic and clinical parameters associated with giardiasis. Odds ratio (OR) estimates with their 95% confidence interval (CIs) and *P* values were calculated (Table 1). $P \leqslant 0.05$ was considered statistically significant. All *P* values were two sided.

RESULTS

Patients

Two hundred and twenty patients fulfilled the criteria. There were 44% (96/220) patients who were giardiasis positive and 56% (124/220) were giardiasis negative. In patients with giardiasis, 72% (69/96) were males and 28% (27/96) were females. The mean age of patients with giardiasis was 28±17 years, while that of patients without giardiasis was 40±18 years ($P<0.001$). Abdominal pain was present in 71% (68/96) patients with giardiasis and in 56% (69/124) patients without giardiasis ($P = 0.02$), diarrhea was present in 29% (28/96) patients with giardiasis and in 14% (17/124) patients without giardiasis ($P = 0.005$, Table 1).

Table 1 Clinical details of patients with and without endoscopic giardiasis

Variables	Giardiasis positive (n = 96) (%)	Giardiasis negative (n = 124) (%)	*P*	OR (95%CI)
Age groups (yr)				
<22	34 (35)	21 (17)		1
22–31	29 (30)	27 (22)		0.7 (0.3–1.5)
32–48	20 (21)	35 (28)		0.4 (0.2–0.9)
>48	13 (14)	41 (33)	<0.001	0.2 (0.1–0.5)
Gender				
Female	27 (28)	43 (35)		
Male	69 (72)	81 (65)	0.301	1.4 (0.8–2.4)
Abdominal pain	68 (71)	69 (56)	0.021	1.9 (1.1–3.4)
Diarrhea	28 (29)	17 (14)	0.005	2.6 (1.3–5.1)
Weight loss	9 (9.4)	6 (5)	0.186	2.0 (0.7–5.9)
Endo-duodenitis	24 (25)	53 (43)	0.007	0.4 (0.2–0.8)
Histological duodenitis	65 (68)	58 (46)	0.002	2.5 (1.4–4.4)
Lamina propria inflammation	73 (76)	53 (42)	<0.001	4.4 (2.4–8.1)
Villus shortening	5 (5)	–	–	–

Results are presented as number (percentage), Odds ratio (95%CI).

Stool examination

Stool examination was carried out in 88.5% (85/96) cases, 6% (5/85) were positive for giardia cysts and trophozoites.

Endoscopy

On EGD, antral gastritis was present in 46% (44/96) patients with giardiasis and in 75% (93/124) patients without giardiasis ($P<0.001$); duodenitis was present in 25% (24/96) patients with giardiasis and in 50% (53/124) patients without giardiasis ($P = 0.006$); duodenal ulcer was present in 4% (4/96) patients with giardiasis and in 72% (89/124) patients without giardiasis ($P<0.001$, Table 1).

Histopathology

On histopathology of gastric antral biopsy, *H. pylori* was seen in 42% (40/96) patients with giardiasis and in 54% (67/124) patients without giardiasis ($P = 0.06$); antral gastritis was found in 53% (51/96) patients with giardiasis and in 81% (100/124) patients without giardiasis ($P<0.001$). Duodenitis was seen in 68% (65/96) patients with giardiasis and in 46% (58/124) patients without giardiasis ($P = 0.002$); villus shortening was seen in 5% (5/96) patients with giardiasis and absent in patients giardiasis negative; lamina propria inflammatory infiltration with increased intraepithelial lymphocytes and plasma cells was seen in 76% (73/96) patients with giardiasis and in 42% (53/124) patients without giardiasis ($P<0.001$, Table 1).

DISCUSSION

It is sometimes difficult to establish the diagnosis of giardiasis. Tests for parasitic antigen in stool are at least as sensitive and specific as good microscopic examination and are easier to perform. All these methods occasionally yield false-negative results.

The implications of this study are that no symptom complex is associated with the giardiasis. Giardiasis may present with abdominal pain alone and it should be considered even in the absence of diarrhea. Abdominal pain is the most common presentation and diagnosis, and investigations carried out are more often for gastritis or peptic ulcer disease with or without *H pylori* infection. Abdominal pain has also been previously found to be significantly associated with the presence of giardiasis[8]. In our study, the incidence of giardiasis was higher in males, which is in agreement with the other studies[9-11]. However, the mean age of our patients was lower and might be attributed to more frequent exposure to this water borne infection because of social activities that involve frequent restaurants beside others. Diarrhea was seen in only 29% of cases. *G lamblia* infestation or other infectious causes were not considered because of the absence of diarrhea in most of these cases and hence a single stool examination was carried out. It is generally held that giardiasis may present with atypical gastrointestinal symptoms but this would be expected in combination with diarrhea[12]. The sensitivity for examination of a single random stool specimen is only 30-50%[13-14]. Hence this might explain the 6% low yield of stool examination in the cases for giardia cysts and trophozoites. Microscopic examination of a single stool sample cannot exclude *G lamblia* infection; therefore, at least three stool specimens should be examined before other diagnostic procedures. As only saline or Lugol's iodine examination of fecal smear was employed, it may have been insufficient in the absence of numerous parasites[14]. Other useful methods such as zinc sulfate floatation, a concentration technique for cysts, were also not employed in our study.

Duodenal ulcer is not a feature of giardiasis, while duodenitis was seen in 25% on endoscopy and in 68% on histology ($P = 0.002$). EGD with duodenal biopsy helps ruling out peptic ulcer as the only cause of symptoms. Giardiasis was associated with the normal endoscopic findings in the duodenum in 75% cases (Table 1). Hence duodenal biopsy should be considered with EGD in patients with abdominal pain as antral biopsy alone may prove inadequate for determining the cause of abdominal pain in a developing country. This is in agreement with the opinion held by some investigators that routine duodenal biopsies should be done in patients undergoing upper intestinal endoscopy[15,16]. In this study, most of our cases on EGD showed normal duodenal mucosa but duodenal biopsy with H&E staining demonstrated *G lamblia* trophozoites (Table 1). Although special stains such as Giemsa or phosphotungstic acid hematoxylin can occasionally be helpful, routine hematoxylin and eosin staining is almost always satisfactory[17].

The data suggesting the role of giardiasis in dyspeptic patients are in contradiction to a previous prospective study[17]. Carr *et al*.[18] carried out a study in an area which is not endemic for Giardia, and demonstrated Giardia in 15.5% of patients presenting with dyspepsia and its prevalence is similar with or without obvious lesions at endoscopy. In their study, only 52% patients presented with abdominal pain, and patients with vomiting and diarrhea as presentation had a significantly increased prevalence of Giardia. However, our data originates from a developing country located in an area endemic for the acquisition of Giardia. In our study, patients more commonly presented with abdominal pain than diarrhea (Table 1). The discrepancy describing a lower frequency of endoscopic duodenitis and higher frequency of histologic duodenitis in infected patients than in non-infected group might be due to a higher prevalence of *H pylori* infection in the latter. However, this was a retrospective observational study with its selection bias and limitations in diagnostic methods. Serial stool examinations were not carried in all patients.

The presentation of giardiasis varies and for diagnosis it requires a high degree of suspicion in the appropriate clinical setting. Examination of duodenal biopsy from patients presenting with abdominal pain should be considered so as to prevent missing diagnosis of giardiasis. Giardiasis is diagnosed more often on EGD with duodenal biopsy rather than on stool examination. As cyst excretion is variable and may be undetectable at times, repeated

examination of properly preserved stool samples and biopsy of the small intestine may be required to detect the parasites. A prospective study is under way to confirm the results of this study.

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• *BRIEF REPORTS* •

Plasma carnitine ester profile in adult celiac disease patients maintained on long-term gluten free diet

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Abstract

AIM: To determine the fasting plasma carnitine ester in patients with celiac disease.

METHODS: We determined the fasting plasma carnitine ester profile using ESI triple quadrupol mass spectrometry in 33 adult patients with biopsy-confirmed maturity onset celiac disease maintained on long term gluten free diet.

RESULTS: The level of free carnitine did not differ as the celiac disease patients were compared with the healthy controls, whereas the acetylcarnitine level was markedly reduced (4.703 ± 0.205 *vs* 10.227 ± 0.368 nmol/mL, $P<0.01$). The level of propionylcarnitine was 61.5%, butyrylcarnitine 56.9%, hexanoylcarnitine 75%, octanoylcarnitine 71.1%, octenoylcarnitine 52.1%, decanoylcarnitine 73.1%, cecenoylcarnitine 58.3%, lauroylcarnitine 61.5%, miristoylcarnitine 66.7%, miristoleylcarnitine 62.5% and oleylcarnitine 81.1% in the celiac disease patients compared to the control values, respectively ($P<0.01$).

CONCLUSION: The marked decrease of circulating acetylcarnitine with 50-80 % decrease of 11 other carnitine esters shows that the carnitine ester metabolism can be influenced even in clinically asymptomatic and well being adult celiac disease patients, and gluten withdrawal alone does not necessarily normalize all elements of the disturbed carnitine homeostasis.

Key words: Plasma carnitine ester profile; Celiac disease

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INTRODUCTION

The adult celiac disease (CD) is a complex autoimmune type of gastrointestinal disorder which can be induced by gluten as a nutritional etiological factor in genetically susceptible persons[1,2]. Metabolism of lipids and lipoproteins is disturbed in the disease[3-7]. The therapy includes withdrawal of the alimentary gluten, introduction of the diet usually results in dramatic clinical improvement and normalization of numerous metabolic deteriorations[1-3]. However, in the case of certain nutriments the diet alone is not enough and supplementation is also necessary.

The primary biochemical function of carnitine is related to its ester-forming capability[8]. In addition to its involvement in ß-oxidation of the long-chain fatty acids, it can form ester with several medium- and short-chain endogenous or exogenous fatty acids[8,9]. In mammals, the body stores of carnitine have exogenous and endogenous origin[10,11]. Several lines of evidence suggest that in human carnitine should be considered as a vitamin-like compound, since the majority of the body stores are of exogenous origin[10,12,13]. The sites of absorption are located in the small intestine[14,15]. These considerations prompted us to obtain information on plasma carnitine esters in patients with CD using tandem mass spectrometry profiling.

MATERIALS AND METHODS

Patients

We examined 33 patients with classic form of celiac disease (9 males, 24 females, mean age: 32.2 ± 2.5 years) and 35 carefully selected clinically healthy age, sex, weight and height matched control subjects (22 males, 13 females, mean age: 31.0 ± 1.9 years; Table 1).

The diagnostic criteria of established CD in our patients included: verification of the specific histological features in small intestinal biopsy specimens, according to

Table 1 Selected clinical and laboratory parameters of patients with celiac disease and control subjects (means ± SE)

	Celiac disease patients $n = 33$		Controls $n = 35$
Females/males	24/9		13 / 22
	at diagnosis	*in current study*	
Age (yrs)	27.4 ± 3.0	32.2 ± 2.5	31.0 ± 1.9
Iron (μmol/L)	13.0 ± 1.5[a]	17.6±1.3[c]	23.1 ± 2.1
Hb (g/dL)	12.6 ± 0.4[a]	13.9±0.3[c]	15.8 ± 0.5
MCV (fL)	85.5 ± 1.7[a]	88.4±1.0[c]	94.3 ± 2.7
RDW (%)	15.7 ± 0.6	14.4±0.4	13.9 ± 0.5
BMI (kg/m^2)	20.0 ± 0.7[a]	22.8±0.6	23.1 ± 1.1

[a]$P < 0.05$ *vs* same group and controls at the time of the study. [c]$P < 0.05$ *vs* controls

the modified Marsh classification[16], positive serological results (antiendomysial antibody and tissue transglutaminase), unequivocally favorable clinical response to the administration of gluten free diet. Patients with any of the rare manifestations of the disease were excluded. All the CD patients were at least 17 years old upon diagnosis, and adhered to gluten free diet for at least one year. All patients received long-term oral iron replacement therapy. Exclusion criteria in both groups were as follows: secondary causes of intestinal atrophy, systemic diseases, any malformations, endocrine disorders, consumption of any drugs, evidence of intestinal bacterial infection, history or evidence for any inherited metabolic disease including those with impairment of glucose and lipid metabolism, smoking, hepatic or renal disease, and pregnancy.

The clinical and laboratory data from the time of diagnosis were from the records of the patients, while the actual results of the current study were from measurements performed from sample aliquots of a blood collection done after an overnight fast precisely between 8:00 and 8:30 AM, both in the celiac disease patients and in the healthy control subjects. This strict postailmental time scheduling was introduced to prevent the diet or fasting time induced dynamic changes of carnitine esters in the circulation[17].

Informed consent was obtained from each participant of the study and the study design was approved by the departmental ethics committee.

Methods

Plasma calcium, iron and albumin levels were determined by routine methods. The blood pictures, including hemoglobin (Hb), mean corpuscular volume (MCV), red blood cell distribution width (RDW) were measured by automated analysis (SYSMEX XE 2100, Japan). The body mass index (BMI) was calculated as body weight/height^2 (in kilograms/m^2).

Acylcarnitines were analyzed as butyl esters using a Micromass Quattro Ultima ESI triple-quadrupole mass spectrometer, combined with a Waters 2795 HPLC system for sample introduction. The procedure was a modified method described previously by Vreken *et al*.[18]. Essentially, 10 μL plasma was first spotted and dried onto a filter paper, then the plasma dot was excised and the excised piece was placed into an Eppendorf tube. Then 200 μL of methanolic stock solution of internal deuterated standards (containing 0.76 μmol/L [^{2}H$_3$]-free carnitine, 0.04 μmol/L [^{2}H$_3$]-propionylcarnitine, 0.04 μmol/L [^{2}H$_3$]-octanoylcarnitine and 0.08 μmol/L [^{2}H$_3$]-palmitoylcarnitine) was added. After 20 min of agitation the supernatant was dried under nitrogen at 40 °C. Derivatization was carried out at 65 °C for 15 min with an addition of 100 μL 3mol/L butanolic HCl. The resulting mixtures were dried again under nitrogen at 40 °C and redissolved in 100 μL mobile phase (acetonitrile:water 80:20). With the help of the autosampler 10 μL of sample aliquots was injected into the mass spectrometer. During the ESI-MS/MS analysis free carnitine and acylcarnitines were measured by positive precursor ion scan of m/z 85, with a scan range of m/z: 200-550. The applied capillary voltage, cone voltage and collision energy were 2.52 kV, 55 V and 26 eV, respectively. The flow rate was 100 μL/min and the total analysis time was 4 min per sample. For each sample the measurements were performed in triplicates beginning with the injection step and the means of the three determinations were used for further calculations.

For statistics Student's t test for unpaired samples was used. The values were expressed as means ± SE, in three decimals for the carnitine esters with respect to the low levels of the long-chain carnitine esters.

RESULTS

Major clinical and laboratory parameters, including those regarded generally as activity markers of CD[19-23] are shown in Table 1. The levels of plasma iron and Hb, and the value of MCV and BMI determined at the time of diagnosis were significantly lower in patients with CD as compared either to the values of the CD patients in the present study, or to the control subjects. In the current study all the previous parameters increased compared with the initial values, but decreased for the plasma iron, Hb and MCV (Table 1).

The plasma circulating carnitine ester profiles are shown in Table 2. The plasma level of free carnitine did not differ between CD patients and controls. By contrast, a marked decrease was found in the acetylcarnitine level in CD patients, which corresponded to 46% of the control value. A significant decrease was also found in the levels of propionyl- (61.5%), butyryl- (56.9%), hexanoyl- (75%), octanoyl- (71.1%), octenoyl- (52.1%), decanoyl- (73.1%) cecenoyl- (58.3%), lauroyl- (61.5%), miristoyl- (66.7%), miristoleyl- (62.5%) and oleylcarnitine (81.1%) in the CD patients as compared with the controls (the rates of decrease are expressed throughout as percent in parentheses taking the controls as 100%).

As a result of the decrease of individual carnitine esters, the plasma level of total esters was lower in CD patients than in controls (6.087 ± 0.571 *vs* 12.166 ± 0.978, $P<0.001$). The ratio of acetylcarnitine/total carnitine esters was 0.773 in the patients and was 0.841 in the controls.

DISCUSSION

We found a marked decrease in acetylcarnitine concentration and a significant decrease in the level of 11 further carnitine esters in plasma of CD patients on long-term gluten free diet. The pattern of the carnitine ester profile found in our patients differs from that found during fasting[17] and differs from the features seen in any of the known metabolic diseases[18,24,25]. The changes observed in the present study could be the result of impaired carnitine homeostasis, consequence of influenced metabolism of the acyl groups derived mainly from the fatty acid metabolism, and combination of thereof.

Damage of the intestinal mucosa can play a central role in the events leading to the changes observed in the current work. Majority of the carnitine reserves are derived from alimentary sources[26,27], the site of the absorption is located in the small intestines[14,15]. The epithelial cells are actively involved in the carnitine- and carnitine ester: contain different carnitine acyltransferases[8] such as the OCTN2 carnitine transporter[28] and the first three enzymes of the mammalian carnitine biosynthesis[29]: trimethyllysine hydroxylase, EC 1.14.11.8; hydroxytrimethyllysine aldolase, EC 4.1.2.'X'; and trimethylaminobutyraldehyde dehydrogenase, EC 1.2.1.47. On the other hand, the mucosa in the small intestine participates in the absorption of triglycerides and plays a complex role in the metabolism of lipoproteins, including chylomicrons, very-low-density lipoproteins, high-density lipoproteins and various apolipoproteins[30-34]. The mucosal damage in CD is classically known to cause fat malabsorption[1,2]. Untreated patients with the classic form of celiac disease may be malnourished and have impaired dietary substrate utilization, including impairment of the metabolism of fats and lipoproteins. It should be noted, that the long-term gluten free diet leads to improvement of several parameters of lipid metabolism[3]. However, the recovery is not necessarily complete for a number of metabolites of lipid metabolism[35,36].

Paradoxically, though the knowledge is growing on the circulating carnitine ester profile features in various disease conditions, very little is known about its normal regulation. Carnitine releases into the circulation by the liver primarily as acetylcarnitine[37] and the actual ester pattern is a result of the uptake/release action of the peripheral tissues. In the present study mainly the short-chain and medium chain carnitine esters were affected. Except for the propionylcarnitine, which can be also derived from the catabolism of amino acids methionine, valine and isoleucine, these acyl groups are mainly degradation products of the longer chain fatty acid oxidation[17], altered profile of the esters found in our asymptomatic patients likely reflects the still affected fatty acid metabolism.

Lipid and lipoprotein metabolism has been extensively investigated in CD, but carnitine homeostasis has hardly been studied. In 1994, Lerner *et al*.[38] investigated the carnitine concentrations in sera of pediatric CD subjects, and found that the total serum carnitine concentration is decreased in patients with active disease as compared with

Table 2 Plasma carnitine ester profiles in celiac disease patients and controls (mean±SE, μmol/L)

	Patients $n = 33$	Controls $n = 35$
Free carnitine (C0)	27.191 ± 1.194	30.029 ± 1.902
Short-chain acylcarnitines		
Acetylcarnitine (C2)	4.703 ± 0.205[b]	10.227 ± 0.368
Propionylcarnitine (C3)	0.247 ± 0.014[b]	0.400 ± 0.021
Butyrylcarnitine (C4)	0.152 ± 0.011[b]	0.267 ± 0.013
Isovalerylcarnitine (C5)	0.111 ± 0.010	0.138 ± 0.010
Tiglylcarnitine (C5:1)	0.034 ± 0.002	0.033 ± 0.003
Medium-chain acylcarnitines		
Hexanoylcarnitine (C6)	0.060 ± 0.004[b]	0.080 ± 0.006
Octanoylcarnitine (C8)	0.086 ± 0.006[b]	0.121 ± 0.009
Octenoylcarnitine (C8:1)	0.037 ± 0.003[b]	0.071 ± 0.008
Decanoylcarnitine (C10)	0.103 ± 0.008[b]	0.141 ± 0.009
Cecenoylcarnitine (C10:1)	0.063 ± 0.005[b]	0.108 ± 0.010
Lauroylcarnitine (C12)	0.032 ± 0.002[b]	0.052 ± 0.004
Long-chain acylcarnitines		
Myristoylcarnitine (C14)	0.016 ± 0.001[b]	0.024 ± 0.001
Myristoleylcarnitine (C14:1)	0.025 ± 0.002[b]	0.040 ± 0.004
Palmitoylcarnitine (C16)	0.097 ± 0.006	0.113 ± 0.006
Palmitoleylcarnitine (C16:1)	0.037 ± 0.003	0.032 ± 0.002
Stearoylcarnitine (C18)	0.076 ± 0.004	0.080 ± 0.004
Oleylcarnitine (C18:1)	0.137 ± 0.007[b]	0.169 ± 0.008
Hydroxymyristoylcarnitine (C14OH)	0.007 ± 0.001	0.005 ± 0.001
Hydroxypalmitoylcarnitine (C16OH)	0.022 ± 0.001	0.023 ± 0.002
Hydroxypalmitoleylcarnitine (C16:1OH)	0.026 ± 0.002	0.029 ± 0.002
Hydroxyoleylcarnitine (C18:1OH)	0.016 ± 0.002	0.013 ± 0.002

[b]$P < 0.01$ *vs* controls

normal subjects, while it was unchanged in CD patients with gluten withdrawal - associated non-active disease[38]. The decrease of carnitine reserves in active disease is likely secondary to the mucosal injury associated damage of the absorption. Albeit similar study on adult subjects is not presented in the literature, after this single pediatric paper the possible development of carnitine deficiency in untreated CD has become widely accepted[39].

In our patients the decrease of total carnitine esters could also reflect shortening of the reserves. It is known that even in patients strictly adhering to a gluten free diet the recovering mucosa can exhibit functional limitations. Therefore, carnitine absorption can be influenced on the one side. On the other side, the mucosa also participates in the trimethyllysine-butyrobetaine conversion, since the first three enzymes of the carnitine biosynthesis are expressed in it[29]. Residual damage can influence this procedure. In addition, trymethyllysine hydroxylase requires Fe^{2+} ion as cofactor[11]. Our patients had improvement after iron replacement therapy, but their plasma iron, Hb and MCV remained decreased. This phenomenon is common in the disease[22]. The decreased tissue iron reserves can also theoretically act on the enzyme activity.

In the recent years there has been increasing recognition that besides the classical major presentations of CD with a malabsorption syndrome and a flat jejunal mucosa, a broad spectrum of metabolic alterations can associate primarily or secondarily with the disease[40,41]. Some of them can be theoretically an early hallmark and predisposing factor for a clinical symptom manifested at a later stage of CD. Inhibition of oxidative metabolism of fatty acids, leading to myopathy with hypotonia and hyporeflexia, hypoglycemia, cardiomyopathy, encephalopathy and disturbed liver function, which are also among the rare extraintestinal manifestation of CD, may be results of carnitine insufficiency[42]. Whether supplementation of carnitine has rationale in the treatment of the disease similar to other metabolic nutriments used routinely in clinical practice[43] remains to be elucidated.

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Investigation of fundo-antral reflex in human beings

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Abstract

AIM: To examine the sensory and motor response(s) of the stomach following fundic distention and to assess whether cholinergic mechanisms influence these responses.

METHODS: Fundic tone, gastric sensory responses and antral motility were evaluated in eight healthy volunteers after a probe with two sensors was placed in the antrum and a highly compliant balloon in the fundus. Isobaric balloon distentions were performed with a barostat. Study was repeated in six volunteers after intravenous atropine was given.

RESULTS: Fundic distention induced large amplitude antral contractions in all subjects. The area under the curve was higher ($P<0.05$) during fundic distention. First sensation was reported at 12±4 mmHg, moderate sensation at 18±4 mmHg and discomfort at 21±4 mmHg. Discomfort was associated with a decrease in antral motility. After atropine was given, the area under the curve of pressure waves and fundic tone decreased ($P<0.05$). Sensory thresholds were not affected.

CONCLUSIONS: Fundic balloon distention induces an antral motor response, the fundo-antral reflex, which in part may be mediated by cholinergic mechanisms.

Key words: Gastric motility; Reflex; Fundo-antral reflex; Sensation

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INTRODUCTION

The stomach performs several important functions. It subserves the function of accommodation and thereby acts as a storage organ. It also functions as a grinder that triturates food into smaller particles and as a pump that transports chyme into the small bowel in a controlled fashion[1]. These functions depend on a complex mix of neurohumoral mechanisms, visceral sensation, intrinsic reflexes, intragastric transport, nutrient composition, particulate size, and the coordinated motor activity of the gastroduodenal unit[2,3].

In ferrets, distension of the corpus produces phasic activity in the antrum, a response termed as excitatory corporo-antral reflex. This is probably mediated by cholinergic mechanisms and intramural gastric pathways[4,5]. Intramural excitatory and inhibitory reflexes have also been demonstrated in isolated gastric preparations[6–9]. Recent studies have suggested that balloon distention of the stomach may increase phasic activity in the antrum and duodenum[10,11]. Whether this phasic activity represents an intrinsic gastro-gastric reflex has not been well characterized.

Furthermore, previous studies have assessed some of the individual components of gastric function[12,13]; whereas an integrated assessment of the biomechanical and sensory properties of the stomach has been scarcely performed. Also, there is very little information regarding the integrated role of the stomach as a sensory, motor and reflex organ. Our hypothesis is that fundic balloon distention may induce reflex antral pressure activity and this response may be mediated by cholinergic mechanisms.

Our objectives were to examine the antral motor responses during step-wise balloon distentions of the fundus, to simultaneously assess the sensory and tone responses of the stomach and to examine if the sensory and motor effects were mediated by cholinergic mechanisms.

METHODS

Study population

Eight healthy volunteers (m/f = 4/4) were recruited for this study. Their mean age was 32±4.95 years. None of them had a history of gastrointestinal or systemic ailments and none was using any medications. All had a normal physical examination. All participants gave written

informed consent and the study protocol was approved by the Human Investigation Review Board of the University of Iowa College of Medicine.

Manometry and barostat assembly

A double lumen plastic-probe (6 mm in diameter) containing a 10 cm long, highly-compliant balloon was used (MUI Scientific; Toronto, ON, Canada). The capacity of the balloon was 600 mL. The balloon was connected to a barostat (GMB Distender II; G&J Electronics Inc., Toronto, Canada). The probe had two perfusion side holes, 5 and 8 cm from the distal end of the balloon. These holes were perfused with gas-free distilled water at a rate of 0.2 mL/min (15 psi) using a low-compliance pneumohydraulic perfusion system (Arndorfer Medical Specialties, Inc., Milwaukee, WI, USA) that connected to transducers (Medex Inc.; MX860-G8618, Hilliard, OH, USA). Intraluminal pressures were relayed to an analog data recorder/amplifier (Medtronics Polygraph, Medtronics Functional Diagnostics; MN, USA) and displayed on a computer monitor using a software program (Polygram for Windows; Synectics Medical AB). The balloon volume and pressure data from the barostat were fed to the polygraph via an interface (Golden Gate; G&J Electronics Inc.).

Thus, the computer display consisted of the intragastric balloon volume, intra-balloon pressure as well as the intraluminal pressure changes in the antrum. The manometric data and the ultrasound images were simultaneously fed into a digital splitter (American Dynamics Ao1479, Orangeburg, NY, USA).

The ultrasonographic image of the cross-sectional diameter of the gastric antrum allowed visualization of antral contractions and assurance that the probe was properly positioned (Acuson 128XP with a 3 mHz sector transducer). The digital splitter synchronized the two images and displayed these images on a monitor screen (VM-17; Javelin, Los Angeles, CA, USA) such that one half of the screen showed the combined manometry and barostat recording and the other half displayed the ultrasound image. These images were recorded on a VHS tape for future analysis.

Study protocol

After an overnight fast, the oropharynx was sprayed with a local anesthetic, pontocaine (Abbott Laboratories, North Chicago, IL, USA). Then the probe with the balloon was placed through the mouth into the stomach. The volunteers were asked to sit in a semi-recumbent position, such that the head end was elevated by 45°. The balloon was distended with 250 mL of air and the probe was slowly retracted until a "tug" was felt signaling that the proximal end of the balloon was located in the fundus. Subsequently, ultrasound images were obtained to check the probe location. The location of pressure sensors in the antrum was also confirmed by the occurrence of typical antral motor pattern consisting of 3-cycle/min activity. The balloon was deflated and the probe was anchored to the cheek with a tape.

After a rest period of 15 min, the balloon was distended by 1 mmHg increments to assess the minimum distending pressure, a pressure at which diaphragmatic oscillations are clearly visible[14]. The intraoperating pressure (IOP) was set at a value of 2 mmHg above the minimum distending pressure using previous criteria[9-11]. Subsequently, a baseline recording of intragastric tone was performed for a period of 20 min. Then isobaric balloon distentions were performed at 3 mmHg increments. Each distention was maintained for 8 min followed by a rest period of 8 min. Thirty seconds after each distention, the subject was asked to rate their sensation on a scale of 0-6 as published previously[14], 0 = no sensation, 1 = vague perception of mild sensation, 2 = definite perception of mild sensation, 3 = vague perception of moderate sensation, 4 = definite perception of moderate sensation, 5 = discomfort, and 6 = pain. If the subject reported discomfort at two incremental distentions or pain at any one distention, the balloon distentions were discontinued. Abdominal ultrasonography was performed intermittently to visualize the antral configuration and morphology. Blood pressure and heart rate were monitored throughout the study.

We administered intravenously 0.6 mg of atropine sulfate in six volunteers (4 m/2 f) after a rest period of 60 min. Five minutes after administration of atropine, the balloon distentions were repeated as described above. Ultrasound images were obtained once again to confirm the location of antral sensors.

Manometric responses

Manometric recordings from the two antral pressure sensors were analyzed visually and manually with the assistance of Polygram for Windows software (Synectics Medical AB). Pressure waves that were $\geqslant$8 mmHg and $\geqslant$3 s in duration were included in the analysis. Artifacts were identified and excluded. There was good quality pressure activity at both channels in approximately 50% of the recordings and therefore an average of the pressure activity at the two antral channels was used. In the rest of the recordings, the pressure activity was more prominent in one of the antral channels and this channel was used for data analysis. The maximum amplitude, duration and area under the curve of each wave were calculated. We also measured the time interval between balloon distention and the onset of the first antral pressure wave as well as the total number of propagating pressure waves in the antrum during each inflation and deflation periods. Propagating waves were defined as pressure waves, which migrated across the antral leads within 6 s of each other and were categorized as either antegrade or retrograde depending on which of the two leads they first appeared in. A similar analysis of the antral pressure waves was performed after atropine injection.

Barostat responses

Gastric tone was assessed by measuring the area under the curve of the gastric volume during isobaric balloon

distentions as previously described[15-17]. The tone changes during and after distention were compared. Likewise, the tone changes obtained during the baseline study were compared with those after atropine injection.

Visceral sensory responses

During intragastric balloon distention, the minimum distending pressure that induced the first perception (a sensation of fullness and discomfort) were calculated. Likewise the sensory responses obtained after administration of atropine were compared to those obtained during the baseline study.

Statistical analysis

The data were presented as mean±SE. The number of pressure waves in the antrum during and after each balloon distention as well as before and after atropine injection was compared using Student's *t*-test. The thresholds for sensory perception and the gastric tone changes before and after atropine injection were compared using ANOVA.

RESULTS

Effects of fundic distention on antral pressure activity

Fundic balloon distention induced antral pressure waves, typically with an amplitude of ⩾50 mmHg (Figure 1). Occasionally, the distention-induced pressure activity persisted for several seconds even after the balloon was deflated, but in most instances the pressure activity ceased after deflation. Incremental balloon distention was associated with a steady increase in antral motility of up to 15 mmHg, but thereafter and particularly at 18 mmHg pressure there was a decrease in antral motility (Figure 2). Interestingly, during successive deflation periods, there was a trend towards progressive decrease in the area under the curve of pressure waves possibly reflecting a recovery of muscle tone.

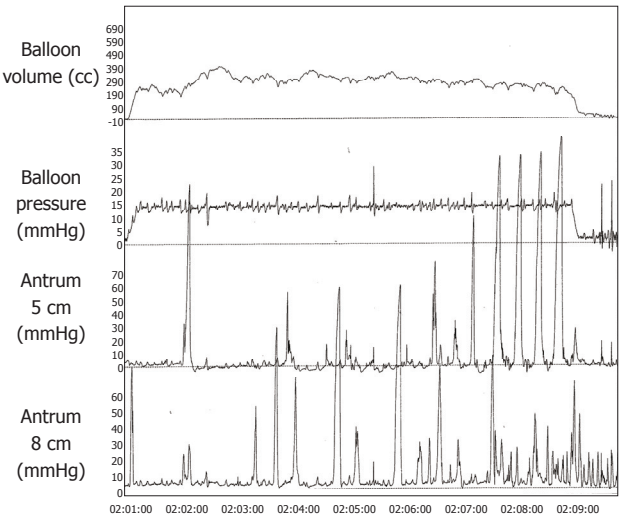

Figure 1 Typical example of the fundo-antral reflex. Channel 1: the fundic balloon volume; channel 2: the balloon pressure; channels 3 and 4: the pressure changes in the antrum.

The mean amplitude of antral pressure waves was also higher ($P<0.05$) during balloon distention than during balloon deflation. For example at balloon distending pressures of 6, 12, and 15 mmHg, the amplitudes were (inflation *vs* deflation) 60(11) *vs* 42(9), 70(14) *vs* 33(7), and 65(11) *vs* 23(7) mmHg respectively. The area under the curve (AUC) of the pressure waves was also significantly higher ($P<0.05$) during balloon distention (Figure 2A).

Visceral sensory responses

The subjects reported a first sensation at distending pressures ranging from 6 to 15 mmHg, a definite perception between 6 and 18 mmHg, a vague perception of moderate sensation between 11 and 21 mmHg, a definite sensation of moderate fullness between 12 and 24 mmHg and definite discomfort between 15 and 28 mmHg (Figure 2B).

Effects of atropine on gastric motor and sensory function

Visceral sensory responses The thresholds for first perception, fullness, and discomfort tended to be lower after administration of atropine but the difference was not significant (Figure 2C).

Antral pressure activity The area under the curve of pressure waves was significantly lower ($P<0.05$) after administration of atropine, particularly at balloon pressures of 6, 9, and 15 mmHg, but not at higher distending pressures (Figure 2D).

Fundic tone responses After administration of atropine, there was a significant increase ($P<0.05$) in balloon volume (Figure 2E) for the same corresponding level of intra balloon pressure, suggesting a decrease in fundic tone.

Cardiovascular responses The mean heart rate/min increased significantly ($P<0.05$) after administration of atropine during most of the distention except at 18 mmHg. There was no significant change in blood pressure.

DISCUSSION

We found that graded balloon distentions of the fundus induced antral pressure waves starting at thresholds that were not perceived by our healthy volunteers. This response was seen in all of our subjects. The area under the curve of pressure waves was significantly higher during the balloon inflation period than during the baseline period or the deflation period. Typically, the waves were ⩾50 mmHg in amplitude. Ultrasound images confirmed that these pressure events were often lumen-occluding contractions. The contractions began within a few seconds after balloon distention. These features suggest the existence of an excitatory gastro-gastric reflex in human beings, wherein distention of the fundus induces antral contractions. In a previous uncontrolled pilot study, we showed that fundic balloon distention may induce antral and duodenal phasic activity[10]. However, in the previous study, gastric visceral sensation or tone was not assessed and likewise the possible role of cholinergic mechanism(s) was not explored.

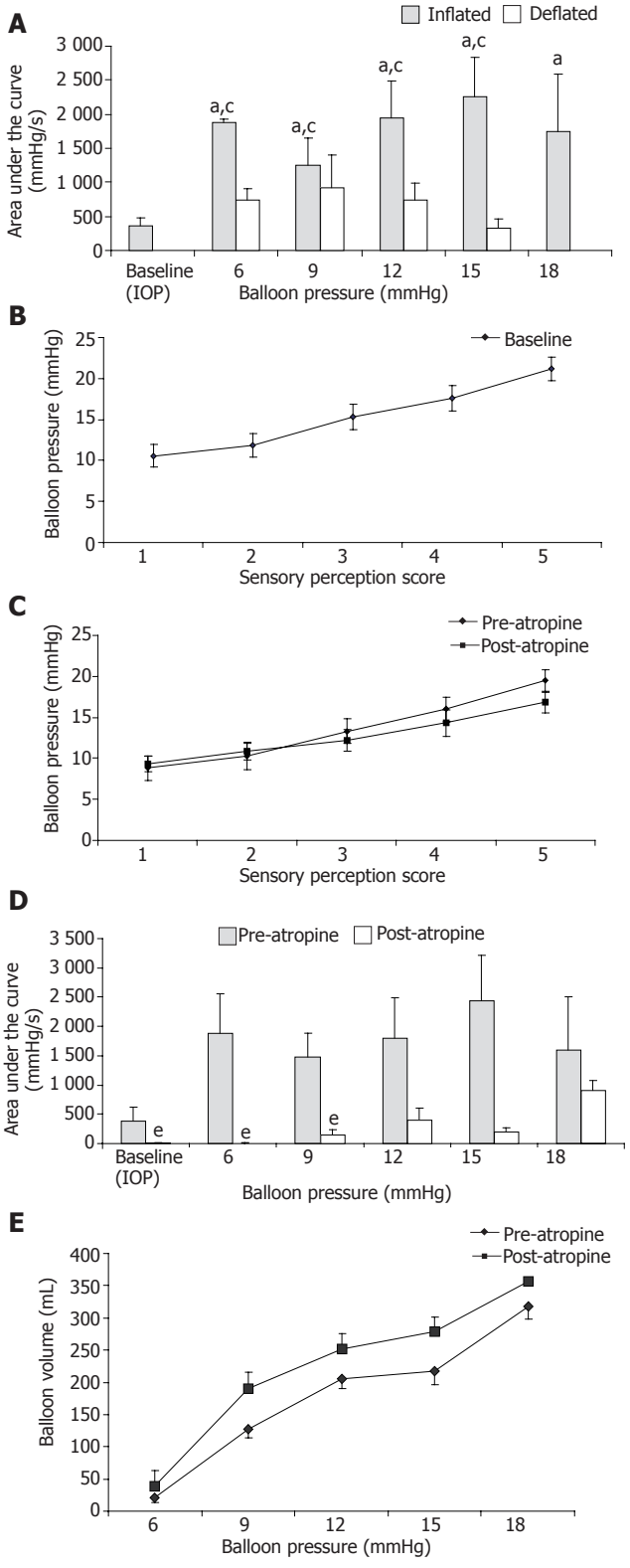

Figure 2 [a]*P*<0.05 inflation *vs* deflation. [c]*P*<0.05 baseline *vs* balloon inflation, [e]*P*<0.05 pre- *vs post*-atropine (**A-E**).

One of the limitations of our study is that we recorded motility from only two antral pressure sensors and it is possible that some of the antral activities may have been missed. However, the number of distention-induced contractions and the area under the curve of pressure waves gradually increased to a distending pressure of 15 mmHg, but thereafter their incidence declined, suggesting that there appears to be some correlation of antral motor responses with the visceral sensory responses. All of our subjects tolerated balloon distentions up to a pressure of 15 mmHg above IOP. Beyond this level of distention, some subjects reported discomfort or pain which was associated with a decrease in pressure activity. Thus, it appears that fundic distention at either subthreshold levels of perception or at thresholds that produce first sensation or fullness may induce reflex antral contractions, whereas higher distending pressures that induce discomfort or pain may cause an attenuation of this response.

After administration of atropine, there was a significant increase in heart rate and decrease in the resting gastric tone. Furthermore, the antral motor activity was also significantly attenuated, particularly at lower levels of balloon distention. At higher distending pressures ($\geqslant$12 mm Hg), some antral pressure activities were seen, though its incidence was lower than those observed before administration of atropine, suggesting that there is an adequate anti-cholinergic response and that the fundo-antral reflex may be partially mediated by cholinergic mechanisms[15]. However, we used a single dose of atropine and it is possible that over time there may have been a loss of anti-cholinergic effect. Also, there was no placebo arm, which is a limitation of this study. Nonetheless, these features suggest that either neuronal or drug-induced inhibition of cholinergic neurotransmission may partly affect gastric motor function. The sensory thresholds were either unchanged or somewhat decreased after administration of atropine, suggesting that gastric sensory responses may not be affected by cholinergic mechanisms, though the study was underpowered to assess this more completely.

Our study showed the possible existence of an intrinsic gastro-gastric reflex that can be induced by fundic distention. This response is different from the fundic relaxation that can be induced by antral distention[7,8] and appears to be partially mediated by cholinergic mechanisms and may in part be related to gastric sensation. Furthermore, unlike the Starling's Law which states that distention of an intestinal segment is associated with proximal contraction and distal relaxation, our result shows that in the human stomach, distention of the fundus is not necessarily associated with antral relaxation. Whether this response plays a role in the trituration of food or in the transport of gastric contents remains to be examined. Also, whether an attenuated or absent fundo-antral reflex plays a role in the pathogenesis of diabetic gastroparesis or functional dyspepsia remains to be explored.

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• *BRIEF REPORTS* •

Validation of four *Helicobacter pylori* rapid blood tests in a multi-ethnic Asian population

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Abstract

AIM: To validate the accuracy of four rapid blood tests in the diagnosis of *Helicobacter pylori*.

METHODS: Consecutive dyspeptic patients scheduled for endoscopy at the National University Hospital, Singapore, were interviewed and had blood drawn for serology. The first 109 patients were tested with BM-test (BM), Pyloriset Screen (PS) and QuickVue (QV), and the next 99 subjects were tested with PS and Unigold (UG). Endoscopies were performed blinded to rapid blood test results and biopsies were taken for culture and rapid urease test. Urea breath tests were performed after endoscopies. The rapid blood test results were compared with four reference tests (rapid urease test, culture, serology, and breath test).

RESULTS: The study population composed of 208 patients (mean age 43.1 years; range 18-73 years; 119 males; 174 Chinese). The number of evaluable patients for BM, QV, UG and PS were 102, 102, 95, and 197, respectively. The sensitivity and specificity, respectively were: PS 80.2%, 95.8%; UG 55.9%, 100%; QV 43.3%, 100%; BM 67.2%, 97.1%.

CONCLUSION: The rapid blood test kits showed high specificity and positive predictive value (97-100%), while sensitivity and negative predictive value ranged widely (43%-80% and 47%-73%, respectively). Among test kits, PS showed the best sensitivity (80%), best negative predictive value (73%) and best negative likelihood ratio (0.207). PS had a specificity of 96%, positive predictive value of 97% and positive likelihood ratio of 19.1.

Key words: *Helicobacter pylori*; Rapid blood test

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INTRODUCTION

There are a variety of methods available for the detection of *Helicobacter pylori (H pylori)*, but many of these are invasive (such as biopsies for rapid urease test, culture, histology, and polymerase chain reaction) or require laboratories (such as urea breath test and serology). Recently, *H pylori* rapid test kits have become available. Rapid blood tests detect *H pylori* antibodies in whole or capillary blood, are easy to use, and yield results in a few minutes, making it a convenient point-of-care test for screening *H pylori*.

The 1997 Asia Pacific Consensus Conference on the management of *H pylori* infection[1] recommended that any blood test must be locally validated, with two or more alternative means of testing, before its widespread application.

Rapid blood test kits have not been widely validated in the Asian populations[2-5]. Local validation is important because the performance characteristics of blood test kits and population prevalence of *H pylori* vary in different populations. In Asian countries, the prevalence of *H pylori* infection is generally higher than in the developed Western nations (such as the United Kingdom, Australia, and France)[6]. In addition, the test performance of rapid blood kits may vary because local *H pylori* strains may be different[7,8].

Our prospective study aimed to validate four rapid blood test kits in the diagnosis of *H pylori* infection in a multi-ethnic Asian population. Amongst the Asian studies till date[2-5], none was conducted in a multi-ethnic population, and all were tested with less than four rapid blood test kits.

MATERIALS AND METHODS

Consecutive patients who were referred from general practice or outpatient clinics and scheduled for endoscopy for initial evaluation of dyspepsia at the National University Hospital, Singapore, were included for the

study. Exclusion criteria included patients with known peptic ulcer or gastric cancer, subjects with prior *H pylori* treatment, and those who had taken antibiotics, bismuth or proton pump inhibitors in the previous one month.

At entry, patients were interviewed using a standard questionnaire. Ten cubic centimeter of blood was drawn from each patient for serology. Each of the first 109 patients were tested with BM-test (BM, Boehringer Mannheim, East Essex, UK), QuickVue (QV, Quidel, CA, USA) and Pyloriset Screen (PS, Orion Diagnostica, Espoo, Finland). The kit with the best sensitivity was retained for continued testing in the next 99 patients together with an additional kit, Unigold (UG, Trinity Biotech, NY, USA). Endoscopy was then performed in the routine fashion by experienced endoscopists blinded to earlier results and three antral biopsy specimens were taken from each patient. Two biopsy specimens were sent for culture and one specimen was sent for the rapid urease test.

A ^{13}C urea breath test was performed directly after endoscopy. The technician doing the urea breath test was blinded to the results of the endoscopy. The results from the rapid blood test, rapid urease test, serology, culture, urea breath test and endoscopy were recorded on a standard data form.

The results of the rapid blood tests were compared with four reference tests: serology using HEL-p Test kit (AMRAD Operations Pty. Ltd, Australia), which had been validated locally[9], culture, rapid urease test, and urea breath test. *H pylori* infection was diagnosed, if any two reference tests were positive. If all the four reference tests were negative, it was assumed that infection was absent. Patients with a single positive test out of the four reference tests were classified as having indeterminate results.

Sample size was estimated based on reference tables[10]. Based on sensitivity of 80% and specificity of 90%, absolute precision of 0.10 and confidence interval of 95%, we needed a minimum of 62 *H pylori*-positive and 35 *H pylori*-negative patients.

The study was approved by the Research and Ethics Committee, National University Hospital, Singapore.

RESULTS

The characteristics of recruited patients are described in Table 1. One hundred and nine patients were tested with BM, QV, and PS (102 evaluable, 7 indeterminate results), and the next 99 subjects with PS and UG (95 evaluable, 4 indeterminate results).

Table 2 shows the sensitivity, specificity, predictive values and likelihood ratios of the respective rapid blood tests for *H pylori*. The rapid blood kits tested all showed specificities above 95% and very good positive predictive values exceeding 97%. There was a wide range in sensitivity between 43% and 80%, negative predictive value ranged from 48% to 73%, and negative likelihood ratios ranged from 0.207 to 0.567. PS had the best sensitivity of 80%, the best negative predictive value of 73%, and the best negative likelihood ratio of 0.207. PS had a high specificity of 96%, a good positive predictive value of 97.1% and a high positive likelihood ratio of 19.1.

Table 1 Characteristics of recruited patients

Kits used	PS, QV, BM	PS, UG	All
Number	109	99	208
Male:female	56:53	63:36	119:89
C:I:M:O[1]	91:12:4:2	83:7:6:3	174:19:10:5
Mean age (range)	44.7 (18–73)	41.3 (20–68)	43.1 (18–73)
H pylori positive	67	59	126
H pylori negative	35	36	71
Indeterminate results	7	4	11

[1]C = Chinese; I = Indian; M = Malay; O = Others.

Table 2 Sensitivity, specificity, predictive values, and likelihood ratios of rapid blood test for *H pylori* infection

Performance characteristics	BM	QV	UG	PS
Sensitivity (%)	67.2	43.3	55.9	80.2
Specificity (%)	97.1	99	99	95.8
Positive predictive value (%)	97.8	98	99	97.1
Negative predictive value (%)	60.7	47.9	58.1	73.1
Positive likelihood ratio	23.2	31.2	41.3	19.1
Negative likelihood ratio	0.338	0.567	0.441	0.207

DISCUSSION

Among the kits tested in our study, PS showed the best sensitivity (84%). Our study showed a wide range in the performance characteristics of the rapid tests. This may be attributable to the antigens used[11] or test kit designs.

The same rapid blood test kit might vary in performance between different populations. For example, QV's sensitivity for *H pylori* was 43.3% in our Singapore population, compared with 81% in Europe[12] and 82% in America[13]. These factors make it important that kits are locally tested and validated before use. A meta-analysis had shown that rapid tests are less accurate than reference tests, with sensitivity and specificity averaging 80-85% and 75-80%, respectively[14].

We conducted this study in an institution. For better evaluation of the potential of rapid blood test as a screening method in primary care, local studies conducted in general practice would be needed. Talley *et al.* reported that when used in general practice in Australia, rapid blood test had a sensitivity of 60% and specificity of 90%[15] Data on the performance characteristics of *H pylori* rapid blood test kits in general practice in the Asian population is lacking.

The Maastricht 2-2000 Consensus report[16] recommended a 'test and treat' approach in the primary care for *H pylori* infection. However, there is a strong association between *H pylori* infection and gastric cancer, especially in the Asian population, which has a high incidence of gastric cancer. Therefore, the use of 'test and treat' approach in Asians remains controversial and awaits further study. PS had a good sensitivity and specificity for the detection of *H pylori* infection, with the positive likelihood ratio of above 10, providing convincing diagnostic evidence, and negative likelihood ratio of 0.2, giving a strong diagnostic evidence. PS might therefore be potentially useful for 'test

and referral' strategy in general practice. Our study, which validated the point-of-care rapid blood test kits in a multi-ethnic Asian population, is an important step for future studies in this area.

In conclusion, there was a wide range in the performance characteristics of rapid blood test, making it important for the kits to be tested and validated locally before being used. Of the rapid blood kits tested, the best sensitivity for *H pylori* detection was 80% (PS). The validation of rapid blood test kits in the local population facilitates future studies on the 'test and treat' or 'test and referral' approach in the Asian population.

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• *BRIEF REPORTS* •

Hepatoprotective effects of *Nigella sativa* L and *Urtica dioica* L on lipid peroxidation, antioxidant enzyme systems and liver enzymes in carbon tetrachloride-treated rats

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Abstract

AIM: To investigate the effects of *Nigella sativa* L (NS) and *Urtica dioica* L (UD) on lipid peroxidation, antioxidant enzyme systems and liver enzymes in CCl_4-treated rats.

METHODS: Fifty-six healthy male Wistar albino rats were used in this study. The rats were randomly allotted into one of the four experimental groups: A (CCl_4-only treated), B (CCl_4+UD treated), C (CCl_4+NS treated) and D (CCl_4+UD+NS treated), each containing 14 animals. All groups received CCl_4 (0.8 mL/kg of body weight, sc, twice a week for 60 d). In addition, B, C and D groups also received daily i.p. injections of 0.2 mL/kg NS or/and 2 mL/kg UD oils for 60 d. Group A, on the other hand, received only 2 mL/kg normal saline solution for 60 d. Blood samples for the biochemical analysis were taken by cardiac puncture from randomly chosen-seven rats in each treatment group at beginning and on the 60th d of the experiment.

RESULTS: The CCl_4 treatment for 60 d increased the lipid peroxidation and liver enzymes, and also decreased the antioxidant enzyme levels. NS or UD treatment (alone or combination) for 60 d decreased the elevated lipid peroxidation and liver enzyme levels and also increased the reduced antioxidant enzyme levels. The weight of rats decreased in group A, and increased in groups B, C and D.

CONCLUSION: NS and UD decrease the lipid peroxidation and liver enzymes, and increase the antioxidant defense system activity in the CCl_4-treated rats.

Key words: CCl_4; Nigella sativa L.; Urtica dioica L.; Lipid peroxidation; Antioxidant enzymes; Rat

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INTRODUCTION

Carbon tetrachloride (CCl_4) is one of the oldest and most widely used toxins for experimental induction of liver fibrosis in laboratory animals[1]. This model has been used in various studies on examined the deposition of extracellular matrix in the fibrotic and cirrhotic liver[2,3].

CCl_4 is a selective hepatotoxic chemical agent. CCl_4-induced reactive free radicals initiate cell damage through two different mechanisms of covalent binding to the membrane proteins and cause lipid peroxidation. A number of investigators have utilized this chemical to produce liver cirrhosis in experimental animals[4]. Production of reactive oxygen species and lipid peroxidation induced by iron overload[5], cholestatic injury[6] and intoxication by ethanol[7] and CCl_4[4] is associated with liver fibrosis and cirrhosis. These effects are partially prevented by antioxidant compounds including α-tocopherol[4,8], silymarin[9] and salvianolic acid[10].

The seed of *Nigella sativa* L (NS), an annual *Ranunculaceae* herbaceous plant, has been used traditionally for centuries in the Middle East, Northern Africa, Far East and Asia for the treatment of asthma. NS contains more than 30 of a fixed oil and 0.40-0.45 w/w of a volatile oil. The volatile oil has been shown to contain 18.4-24% thymoquinone and 46% many monoterpenes such as p-cymene, and α-pinene[11]. Recently conducted clinical and experimental researches have shown many therapeutic effects of NS extracts such as immunomodulator[12], anti-inflammatory[13] and anti-tumour agents[14].

Urtica dioica L (UD) is a plant belonging to the plant family *Urticaceae*. Its seeds are widely used in folk medicine in many parts of Turkey, especially in the therapy of advanced cancer patients. Polar extract of the UD contains lignans (+)-neoolivil, (-)-secoisolariciresinol, dehydrodiconiferyl alcohol, isolariciresinol, pinoresinol, and 3,4-divanillyltetrahydrofuran, and has antiinflammatory

effects[15] and stimulates the proliferation of human lymphocytes[16].

The present study aimed to investigate the preventive effects of NS and UD on lipid peroxidation, antioxidant enzyme systems and some liver enzymes in CCl_4-treated rats.

MATERIALS AND METHODS

Plant materials and extraction procedure

The NS and UD seeds were purchased from a local herb store, Zonguldak, Turkey. Voucher specimens were kept at the Department of Biochemistry, Zonguldak Karaelmas University, Zonguldak, Turkey for the future reference. The seeds of NS were powdered in a mixer, placed in a distillation flask and the volatile oil with 0.2 % yield was collected by a steam distillation. The fixed oil of UD was extracted with the help of a rotary evaporator using diethyl ether as solvent.

Treatment of rats

Fifty-six male Wistar albino rats, weighing 150-200 g, averaging 16 wk old, were used in this study. The rats were randomly allotted into one of the four experimental groups: A (CCl_4-only treated), B (CCl_4+UD treated), C (CCl_4+NS treated) and D (CCl_4+UD+NS treated), each containing 14 animals. All groups received CCl_4 (Merck; 153.82 g/moL, 1.59 kg, Germany, 0.8 mL/kg of body weight, sc, twice a week for 60 d). In addition, B, C and D groups also received the daily ip injection of 0.2 mL/kg NS or/and 2 mL/kg UD oils for 60 d. Group A, on the other hand, received only 2 mL/kg normal saline solution for 60 d. The animals were housed in macrolon cages under standard laboratory conditions (light period 7.00 a.m. to 7.00 p.m., 21±1 °C, rat chow and tap water freely available). All animals received human care according to the criteria outlined in the "Guide for the Care and Use of Laboratory Animals" prepared by the National Academy of Sciences and published by the National Institutes of Health. The experiment lasted for 60 d.

Biochemical analysis

Blood samples for the biochemical analysis were taken from each treatment group at beginning and on the 60th d of the experiment. Rats from which blood samples were taken were excluded from the experiment to eliminate the haemorrhage- and stress-induced complications. At the end of the experiment, rats in all groups were starved overnight, and sacrificed under chloralhydrate (6 mL of 7% chloralhydrate/kg, Sigma, St. Louis, MO, USA) anaesthesia. Blood samples were collected by cardiac puncture using heparinised syringes. Leukocytes and plasma components were seperated by centrifugation of the blood. Erythrocytes were washed three times with 0.9% NaCl solution and packed, and then stored at -70 °C until study.

Blood MDA (mmol/L) was determined by the double heating method of Draper and Hadley[17]. The principle of the method is spectrophotometric measurement of the colour produced during the reaction to thiobarbituric acid (TBA) with MDA. For this purpose, 2.5 mL of 100 g/L trichloroacetic acid solution was added to 0.5 mL erythrocytes in each centrifuge tube and placed in a boiling water bath for 15 min. After cooled in tap water, the mixture was centrifuged at 1 000 r/min for 10 min, and 2 mL of the supernatant was added to 1 mL of 6.7 g/L TBA solution in a test tube and placed in a boiling water bath for 15 min. The solution was then cooled in tap water and its absorbance was measured using a Shimadzu UV-1601 (Japan) spectrophotometer at 532 nm. The concentration of MDA was calculated by the absorbance coefficient of MDA-TBA complex 1.56×10^5 /cm, and expressed in μmol/g Hb erythrocytes and μmol/g tissue protein.

Blood GSH concentration was measured by the method described by Beutler *et al.*[18]. Briefly, 200 μL of whole blood was added to 1.8 mL of distilled water. Three ml of the precipitating solution was mixed with the hemolysate. The mixture was allowed to stand for approximately 5 min and then filtered. Two milliliters of filtrate were taken and added into another tube, and then, 8 mL of the phosphate solution and 1 mL of the DTNB [5,5'dithiobis-(2-nitrobenzoic acid)] were added. A blank was prepared with 8 mL of the phosphate solution, 2 mL of the diluted precipitating solution (three parts to two parts of the distilled water), and 1 mL of the DTNB reagent. A standard solution of glutathione was prepared (40 mg/100 mL). The optical density was measured at 412 nm with a spectrophotometer.

Serum ceruloplasmin *p*-phenylenediamine (PPD) oxidase activity was measured according to Sunderman and Nomoto[19]. At pH 5.4, ceruloplasmin catalyze the oxidation of PPD to yield a colored product. The rate of formation of the colored oxidation product was proportional to the concentration of serum ceruloplasmin if a correction was made for nonenzymatic oxidation of PPD.

Vitamin E was analyzed colorimetrically with 2,4,6-tripridyl-s-triazin and $FeCl_3$ after the extraction with absolute ethanol and xylene[20]. Serum vitamin C level was determined after derivatisation with 2,4-dinitrophenylhydrazine[21]. The levels of β-carotene at 425 nm and retinol at 325 nm were detected after the reaction of serum : ethanol : hexane at the ratio of 1 : 1 : 3 respectively[22].

Alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP) levels were determined by an autoanalyzer (Roche-Hittachi, Japan) using commercial kits (Roche, Basel, Switzerland).

Statistical analysis

The data were expressed as mean±SD and analysed by repeated measures of variance. Tukey test was used to test for differences among means when ANOVA indicated a significant ($P\leqslant0.05$) F ratio.

Table 1 Blood MDA level (nmol/mL erythrocytes) in CCl_4+NaCl-treated (A), CCl_4+UD treated (B), CCl_4+NS treated (C) and CCl_4+UD+NS treated (D) rats (mean±SD)

Groups(d)	A	B	C	D
0	1.23±0.09	1.18±0.14	1.21±0.12	1.25±0.03
60	2.81±0.05	1.35±0.07	1.41±0.09	1.28±0.12

Table 2 Serum antioxidant levels (mg/dL) in CCl_4+NaCl-treated(A), CCl_4+UD treated (B), CCl_4+NS treated (C) and CCl_4+UD+NS treated (D) rats (mean±SD)

Groups(d)	A	B	C	D
GSH				
0	50.76±0.72	47.14±1.02	47.72±1.25	49.36±0.93
60	40.21±2.38	55.44±3.32	50.63±3.41	61.12±8.26
Ceruloplasmin				
0	18.72±0.82	18.47±0.89	17.36±1.13	19.23±0.59
60	12.92±0.49	29.33±0.64	25.90±1.61	30.22±1.23
Vitamin E				
0	0.20±0.12	0.19±0.01	0.18±0.01	0.20±0.13
60	0.11±0.02	0.34±0.03	0.24±0.02	0.45±0.01
Vitamin C				
0	0.53±0.01	0.51±0.01	0.52±0.01	0.54±0.01
60	0.40±0.01	0.61±0.02	0.54±0.02	0.89±0.01
Beta-karoten				
0	26.49±0.85	25.28±0.44	26.41±0.37	25.15±0.83
60	19.28±1.23	25.21±1.55	26.73±1.53	25.25±1.27
Retinol				
0	50.33±0.87	52.72±0.66	51.22±0.39	52.64±0.55
60	44.98±1.18	56.19±0.37	53.52±0.63	61.69±1.69

Table 3 Serum liver enzyme levels (U/L) in CCl4+NaCl-treated (A), CCl4+UD treated (B), CCl4+NS treated (C) and CCl4+UD+NS treated (D) rats (mean±SD)

Groups(d)	A	B	C	D
ALP				
0	957±23.62	961±16.64	987±13.64	1028±36.51
60	1354±32.58	692±24.21	637.82±31.21	772.57±91.45
ALT				
0	89.3±4.86	78.21±17.35	80.14±20.12	83.17±11.21
60	2762±42.45	553±60.29	425±42.24	253±39.27
AST				
0	143.29±6.43	141.28±16.26	143.50±7.85	144.27±20.44
60	2342.25±34.06	554.23±14.83	1031.44±92.68	672.17±58.42

Table 4 Weights (g) of rats in CCl_4+NaCl-treated (A), CCl_4+UD treated (B), CCl_4+NS treated (C) and CCl_4+UD+NS treated (D) rats (mean±SD)

Groups(d)	A	B	C	D
0	189±6.21	181.43±12.21	187.21±4.33	189.63±3.21
60	171.73±3.90	204.02±12.09	214.76±12.72	217.50±11.52

RESULTS

The levels of blood MDA, antioxidants of all groups and the levels of serum liver enzymes are shown in Table 1-3, respectively. The CCl_4 treatment for 60 d significantly ($P<0.05$) increased the MDA and liver enzymes, and also decreased ($P<0.05$) the antioxidant levels. NS or UD treatment (alone or in combination) for 60 d significantly ($P<0.05$) decreased the elevated MDA and liver enzyme levels and also increased ($P<0.05$) the reduced antioxidant levels. The weight of rats decreased ($P<0.05$) in group A, and increased ($P<0.05$) in groups B, C, and D (Table 4).

DISCUSSION

Treatment of animals with CCl_4 is known to cause severe hepatic injury[23]. In our study, we showed that repeated CCl_4 treatment for 60 d increased the lipid peroxidation and liver enzymes, and also decreased the antioxidant enzyme levels. It has been suggested that the lipid peroxidation may be a link between tissue injury and liver fibrosis by modulating collagen gene expression[24]. It was reported that CCl_4 is suitable to induce lipid peroxidation in experimental animals within a few minutes after administration and its long-term use results in liver fibrosis and cirrhosis by lipid peroxidation pathway[25]. It is generally thought that CCl_4 toxicity is due to reactive free radical (CCl_3.), which is generated by its reductive metabolism by hepatic cytochrome P450. The reactive intermediate is believed to cause lipid peroxidation and breakdown of cellular membranes[26].

Recent experimental studies have investigated the role of antioxidative vitamins, minerals, drugs and plant-derived compounds in the prevention and therapy of liver fibrosis. Parola *et al*.[4] showed that an increased liver content of vitamin E leads to a significant degree of protection against carbon tetrachloride-induced chronic liver damage and cirrhosis in rats. Ianas *et al*.[27] have described the all-round beneficial action of a selenium preparation upon the organism in rats exposed to CCl_4 as well as a strong antioxidative effect, confirming the essential role of selenium in maintaining cellular integrity.

Several plant derived compounds such as colchicine (Colchicum dispert), silymarin (Slybum marianum), polyenylphosphatidyl choline (soy bean), ellagic acid (cruciferous vegetables), Gingko biloba composita and recently Sho-saiko-to (extract of seven herbs in Chinese folk medicine) have been proposed as antioxidants and antifibrotics in the treatment of chronic liver disease[28-30]. The antioxidative and hepatoprotective effects of chitosan against CCl_4-induced liver toxicity in rats have been under investigation by measuring thiobarbituric acid reactive substances (TBARS) and antioxidant enzyme activities[31]. The antioxidant systems such as antioxidant vitamins (A, C, and E), superoxide dismutase (SOD), catalase, glutathione (GSH), ceruloplasmin and glutathione peroxidase (GSH-Px) protect the cells against lipid peroxidation, which is the base of many pathologic processes [32,33].

In our study, we found that NS or UD treatment (alone or combination) for 60 d decreased the elevated MDA and liver enzyme levels and also increased the reduced antioxidant enzyme levels in CCl_4-treated rats. Previously performed clinical and experimental investigations have shown that NS has a protective effect against oxidative damage in isolated rat hepatocytes[34]. It was found that the fixed oil of NS has both antioxidant and anti-eicosanoid effects greater than thymoquinone which is its active

constituent[13]. Furthermore, NS has antioxidant activity by suppressing the chemiluminescence in phagocytes[35].

Recently, Turkdogan *et al.*[36] observed that NS has a significant hepatoprotective effect in CCl_4-administrated rabbits, ans that hepatocellular degenerative and necrotic changes are slight without advanced fibrosis and cirrhotic process in NS-treated group. However, Turkdogan *et al.*[37] found that NS can prevent liver fibrosis and cirrhosis, suggesting that NS protects liver against fibrosis possibly through immunomodulator and antioxidant activities.

There are no comprehensive studies on the therapeutic effects of UD. Only one study reported that UD extract can inhibit *in vitro* prostate cancer cell proliferation[38]. It has been suggested that the extract of UD is effective in inducing glutathione S-transferase, SOD and catalase activity in the forestomach and SOD and CAT activity in the lung at both dose levels[39]. However, Turkdogan *et al.*[36] showed that NS and UD can significantly prevent CCl_4-induced hepatotoxicity in rats. Our biochemical results demonstrated that NS and UD treatment prevented CCl_4-induced hepatotoxicity in rats by decreasing the lipid peroxidation and increasing the antioxidant defense system activity. However, Kanter *et al.*[40] also showed the NS and UD increase the antioxidant defense system activity in experimentally CCl_4-treated rats.

In conclusion, NS and UD decrease lipid peroxidation and liver enzymes, and increase antioxidant defense system activity in the CCl_4-treated rats. They also prevent weight lose induced by the CCl_4 treatment. Further studies are required to evaluate the possible hepatoprotective effect of NS and UD which are traditionally used as a medicine for many complaints including liver diseases.

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• *BRIEF REPORTS* •

Preoperative sorting of circulating T lymphocytes in patients with esophageal squamous cell carcinoma: Its prognostic significance

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Abstract

AIM: To elucidate the immunologic parameters for the outcome of patients with malignant tumors, especially esophageal squamous cell carcinoma (ESCC) associated with high malignant potential.

METHODS: Clinicopathologic features were compared between patients with lower and higher CD4 and CD8 values as well as CD4/CD8 ratio in peripheral blood.

RESULTS: The survival rate of patients with higher CD4 value was significantly better than that in patients with lower CD4 value (P = 0.039). The survival rate of patients with higher CD8 value was significantly worse than that of patients with lower CD8 value (P = 0.026). Similarly, the survival rate of patients with higher CD4/CD8 ratio was significantly better than that of patients with lower CD4/CD8 ratio (P = 0.042). Additionally, multivariate analysis demonstrated that lower CD8 and lower CD4/CD8 ratio were factors independently associated with worse prognosis of patients.

CONCLUSION: All the immunologic parameters can predict the outcome of patients with ESCC.

Key words: Lymphocyte sub-population; Esophagus; Squamous cell carcinoma; Prognostic indicator

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INTRODUCTION

Impaired immunity is well known to be correlated with the tumorigenesis and/or progressive behavior of human tumors[1-3]. Therefore, it is important to assess the immunologic dynamics of patients with malignant tumors, especially esophageal carcinoma.

We have reported the significance of preoperative assessment of such immunological parameters as serum C-reactive protein concentration[4], prognostic nutritional index[5], and phytohemagglutinin (PHA) response test[6] as a prognostic indicator in esophageal carcinoma.

CD8+, cytotoxic T lymphocytes, plays an immunologic role as the specific tumor terminator and CD4+, helper T lymphocyte, serves the function of controlling CD8+ T-cell-dependent tumor termination[7]. However, only a few investigations are available on the clinicopathologic significance of these lymphocytes in controlling esophageal carcinoma[8-10].

It was reported that lower CD4/CD8 ratio in peripheral blood can be used as an indicator for worse prognosis of patients with esophageal carcinoma[11]. In the current study, we investigated the clinical significance of the serum values of CD4 and CD8, and the CD4/CD8 ratio in patients with esophageal squamous cell carcinoma (ESCC).

MATERIALS AND METHODS

One hundred and thirty-four patients (118 men and 16 women) with ESCC, who underwent esophageal resection and reconstruction of the digestive tract in our institute between 1990 and 1997, were enrolled in this study. The patients had a median age of 62 years (range, 41-82 years).

Follow-up was continued until their death. The interval of follow-up ranged from 29 d to 8 years and 9 mo averaged 2 years and 11 mo. Serum values of lymphocyte sub-populations, CD4 and CD8, were measured as previously described[11].

Pathological features were presented according to the guidelines for clinical and pathologic studies on carcinoma of the esophagus established by the Japanese Society for Esophageal Diseases[12], and clinical stages were determined by the TNM classification of malignant tumors approved by the International Union Against Cancer[13]. Clinicopathologic features were compared between patients with lower and higher values of CD4 and

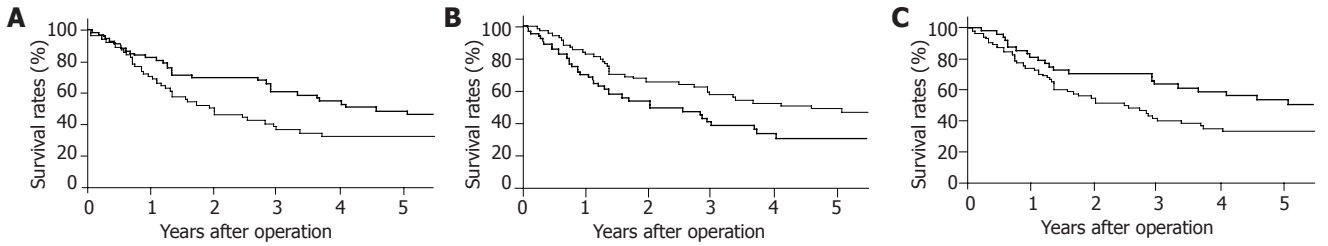

Figure 1 **A:** Survival rate in group H-CD4 (thick line) and group L-CD4 (thin line, *P*=0.039); **B:** Survival rate in group H-CD8 (thick line) and group L-CD8 (thin line, *P*=0.026); **C:** Survival rate in group H-CD4/8 (thick line) and group L-CD4/8 (thin line, *P*=0.042).

Table 1 Relationship between serum CD4 value and clinicopathological features (mean±SD), *n* (%)

Variables	Group H-CD4 (*n* = 68)	Group L-CD4 (*n* = 66)	*P*
Gender			
Male	57 (83.8)	61 (92.4)	0.205
Female	11 (16.2)	5 (7.6)	
Age	60.9±8.8	64.1+9.6	0.046
Location of tumor			
Upper	12 (17.6)	11 (16.7)	0.845
Middle	38 (55.9)	40 (60.6)	
Lower	18 (26.5)	15 (22.7)	
Degree of differentiation			
Well	4 (5.9)	6 (9.1)	0.620
Moderately	57 (83.8)	51 (77.3)	
Poorly	7 (10.3)	9 (13.6)	
Tumor size (cm)	5.3+2.7	5.4+2.5	0.970
Depth of tumor			
Tis	2 (2.9)	2 (3.0)	0.051
T1a	6 (8.8)	5 (7.6)	
T1b	15 (22.1)	9 (13.6)	
T2	14 (20.6)	9 (13.6)	
T3	15 (22.1)	33 (50.0)	
T4	16 (23.5)	8 (12.2)	
Lymph nodes metastasis			
Positive	24 (35.3)	31 (53.0)	0.231
Negative	44 (64.7)	35 (47.0)	
Lymphatic invasion			
Positive	12 (17.6)	18 (27.3)	0.259
Negative	56 (82.4)	48 (72.7)	
Venous invasion			
Positive	5 (7.4)	11 (16.7)	0.163
Negative	63 (92.6)	55 (83.3)	
TNM stage			
0	2 (2.9)	2 (3.0)	0.749
I	17 (25.0)	11 (16.7)	
IIA	16 (23.5)	18 (27.3)	
IIB	10 (14.7)	8 (12.2)	
III	23 (33.8)	27 (40.8)	
Curability			
Curative resection	52 (76.5)	56 (84.8)	0.314
Non curative resection	16 (23.5)	10 (15.2)	

Well = well differentiated squamous cell carcinoma; Moderately = moderately differentiated squamous cell carcinoma; Poorly = poorly differentiated squamous cell carcinoma.

CD8 as well as CD4/CD8 ratio.

Chi-square test and Student's *t* test were used to compare the clinicopathologic data. The cumulative survival rates were calculated by the Kaplan-Meier method and the survival curves were tested by the Mantel-Cox method. $P<0.05$ was considered statistically significant.

RESULTS

Among the clinicopathologic factors, the mean age of patients with higher CD4 value (group H-CD4) was significantly lower than that of patients with lower CD4 value (group L-CD4, $P = 0.046$). However, no significant difference was observed in other factors including tumor-related factors (Table 1). The 1-, 3-, and 5-year survival rates were 82.2%, 60.4% and 48.4%, respectively, in group H-CD4 and 70.8%, 36.9% and 32.7%, respectively, in group L-CD4 ($P = 0.039$, Figure 1A).

No significant difference was found in the clinicopathologic factors between patients with higher (group H-CD8) and lower CD8 value (group L-CD8, Table 2). The 1-, 3-, and 5-year survival rates were 69.7%, 38.5% and 30.0%, respectively, in group H-CD8 and 82.7%, 57.7%, and 49.0%, respectively, in group L-CD8 ($P = 0.026$, Figure 1B).

Significant difference between patients with higher (group H-CD4/8) and lower CD4/CD8 ratio (group L-CD4/8) was observed only in gender proportion ($P = 0.036$, Table 3). The 1-, 3-, and 5-year survival rates were 80.9%, 62.9%, and 52.9%, respectively, in group H-CD4/8 and 74.0%, 40.3% and 33.0%, respectively, in group L-CD4/8 ($P = 0.042$, Figure 1C).

Multivariate analysis demonstrated that lower CD8 (95%CI, 2.07, 1.26–3.38; $P = 0.004$) and lower CD4/CD8 ratio (95%CI, 1.73, 1.02-2.93; $P = 0.043$) were factors independently associated with worse prognosis of patients.

DISCUSSION

With the development of monoclonal antibodies in detecting lymphocytes subpopulation[14], lymphocyte subtypes in peripheral blood were examined to investigate their functions in immune-surveillance. Among the subpopulations of lymphocytes, investigations of cancer immunology have been focused on CD8, suppressor/

Table 2 Relationship between serum CD8 value and clinico-pathological features (mean±SD), *n* (%)

Variables	Group H-CD8 (*n* = 64)	Group L-CD8 (*n* = 70)	*P*
Gender			
Male	58 (90.6)	60 (85.7)	0.543
Female	6 (9.4)	10 (14.3)	
Age	63.4+9.6	61.6+9.1	0.265
Location of tumor			
Upper	9 (14.1)	14 (20.0)	0.635
Middle	38 (59.4)	40 (57.1)	
Lower	17 (26.5)	16 (22.9)	
Degree of differentiation			
Well	4 (6.3)	6 (8.6)	0.810
Moderately	53 (82.8)	55 (78.6)	
Poorly	7 (10.9)	9 (12.8)	
Tumor size (cm)	5.4+2.4	5.3+2.8	0.930
Depth of tumor			
Tis	2 (3.1)	2 (2.8)	0.899
T1a	4 (6.3)	7 (10.0)	
T1b	11 (17.2)	13 (18.6)	
T2	10 (15.6)	13 (18.6)	
T3	26 (40.6)	22 (31.4)	
T4	11 (17.2)	13 (18.6)	
Lymph nodes metastasis			
Positive	29 (45.3)	26 (37.1)	0.382
Negative	35 (54.7)	44 (62.9)	
Lymphatic invasion			
Positive	11 (17.2)	19 (27.1)	0.241
Negative	53 (82.8)	51 (72.9)	
Venous invasion			
Positive	9 (14.1)	7 (10.0)	0.647
Negative	55 (85.9)	63 (90.0)	
TNM stage			
0	2 (3.1)	2 (2.8)	0.858
I	12 (18.8)	16 (22.9)	
IIA	15 (23.4)	19 (27.1)	
IIB	8 (12.5)	10 (14.3)	
III	27 (42.2)	23 (32.9)	
Curability			
Curative resection	51 (79.7)	57 (81.4)	0.830
Non curative resection	13 (20.3)	13 (18.6)	

Well = well differentiated squamous cell carcinoma; Moderately = moderately differentiated squamous cell carcinoma; Poorly = poorly differentiated squamous cell carcinoma.

Table 3 Relationship between serum CD4/CD8 ratio and clinico-pathological features (mean±SD), *n* (%)

Variables	Group H-CD4/8 (*n* = 48)	Group L-CD4/8 (*n* = 86)	*P*
Gender			
Male	38 (79.2)	80 (93.0)	0.036
Female	10 (20.8)	6 (7.0)	
Age	61.4+8.9	63.0+9.6	0.322
Location of tumor			
Upper	10 (20.8)	13 (15.1)	0.675
Middle	26 (54.2)	52 (60.5)	
Lower	12 (25.0)	21 (24.4)	
Degree of differentiation			
Well	5 (10.4)	5 (5.8)	0.459
Moderately	36 (75.0)	72 (83.7)	
Poorly	7 (14.6)	9 (10.5)	
Tumor size (cm)	4.9+2.5	5.6+2.7	0.125
Depth of tumor			
Tis	2 (4.2)	2 (2.3)	0.213
T1a	4 (8.3)	7 (8.1)	
T1b	11 (22.9)	13 (15.1)	
T2	8 (16.7)	15 (17.5)	
T3	11 (22.9)	37 (43.0)	
T4	12 (25.0)	12 (14.0)	
Lymph nodes metastasis			
Positive	16 (33.3)	39 (34.9)	0.241
Negative	32 (66.7)	47 (65.1)	
Lymphatic invasion			
Positive	12 (25.0)	18 (20.9)	0.745
Negative	36 (75.0)	68 (79.1)	
Venous invasion			
Positive	5 (10.4)	11 (12.8)	0.647
Negative	43 (89.6)	75 (87.2)	
TNM stage			
0	2 (4.2)	2 (2.3)	0.858
I	13 (27.1)	15 (17.5)	
IIA	11 (22.9)	23 (26.7)	
IIB	6 (12.5)	12 (14.0)	
III	16 (33.3)	34 (39.5)	
Curability			
Curative resection	36 (75.0)	72 (83.7)	0.830
Non curative resection	12 (25.0)	14 (16.3)	

Well = well differentiated squamous cell carcinoma; Moderately = moderately differentiated squamous cell carcinoma; Poorly = poorly differentiated squamous cell carcinoma.

cytotoxic T lymphocyte responses. Attention has also been paid to CD4, helper/inducer T lymphocytes, as a critical component of the anti-tumor immune response[15].

Tumor-specific immune response depends on the function of activated CD4 cells[16], and therefore the deficiency in the function of activated CD4 cells might be directly correlated with the immune-deficiency of the host. CD4 helper/inducer T lymphocytes produce lymphokines, thus promoting the cytotoxic activity of CD8 T lymphocytes[17,18]. Therefore, activation of both CD4 and CD8 can exert a synergistic immune response to the termination of tumor cells.

Though some investigations have demonstrated an immunologic anti-tumor effect of CD4 and CD8[8], the clinical significance of CD4/CD8 ratio in tumor infiltrating lymphocytes and/or in peripheral blood as an indicator of progressive gastrointestinal tumor and/or worse prognosis of patients has been occasionally reported[19-21]. Diederichsen *et al.*[19] reported that low CD4/CD8 ratio in tumor infiltrating lymphocytes is an independent prognostic indicator in patients with colorectal carcinoma. Decrease of the CD4/CD8 ratio is correlated with progressive behavior of the tumor indicated by such tumor-related factors as stage of the tumor, tumor invasion, lymph node metastasis, and size of the tumor in gastric cancer[20]. Moreover, severe pre-

operative cellular immune-suppression, where CD4/CD8 ratio was less than 1.0, is a predictive parameter for mortality in patients with gastric cancer[21].

CD8 expression in TIL in tumor tissue can serve the function of suppressing the proliferation of ESCC[9], and similarly CD8 infiltration into the tumor is an independent prognostic indicator for ESCC[10]. Recently, increase of the number of CD4 and CD8 T lymphocytes in tumor nests and stroma has been found to be an independent indicator of favorable prognosis of patients with ESCC[8].

These results suggest that CD8 T-lymphocyte infiltration, as have been investigated in some other tumors[22,23], plays a pivotal role in immune-potential against ESCCs.

However, it was reported that the prognosis of patients with lung carcinoma associated with more CD8 expressing T cells within cancer nests is significantly worse than that of patients with tumors of fewer CD8 expressing T cells[24]. High percentage of activated CD8-positive cells in postoperative peripheral blood is an indicator of worse prognosis for renal cell carcinoma[25].

The different methods used to evaluate the value or expression of CD8, histological type of the tumor, or balance between immunologic dynamics of the tumor and the host might explain this possible discrepancy in the significance of CD8 T lymphocytes in anti-tumor immune.

In the current study, the decreased CD4/CD8 ratio as well as the increased CD8 and the decreased CD4 in peripheral blood could predict the worse prognosis in patients with ESCCs. Preoperative coexistence of impaired immunity could influence the postoperative complications[5]. The incidence of postoperative complications is an independent indicator of worse prognosis in patients with esophageal carcinoma[26]. Therefore, preoperative impaired immunity seems not to be negligible as the cause of death, other than esophageal carcinoma.

Assessment of preoperative immunity in patients seems to be of great importance in predicting the subsequent outcome of patients with ESCCs.

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• *BRIEF REPORTS* •

DNA end binding activity and Ku70/80 heterodimer expression in human colorectal tumor

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Abstract

AIM: To determine the DNA binding activity and protein levels of the Ku70/80 heterodimer, the functional mediator of the NHEJ activity, in human colorectal carcinogenesis.

METHODS: The Ku70/80 DNA-binding activity was determined by electrophoretic mobility shift assays in 20 colon adenoma and 15 colorectal cancer samples as well as matched normal colonic tissues. Nuclear and cytoplasmic protein expression was determined by immunohistochemistry and Western blot analysis.

RESULTS: A statistically significant difference was found in both adenomas and carcinomas as compared to matched normal colonic mucosa (P<0.00). However, changes in binding activity were not homogenous with approximately 50% of the tumors showing a clear increase in the binding activity, 30% displaying a modest increase and 15% showing a decrease of the activity. Tumors, with increased DNA-binding activity, also showed a statistically significant increase in Ku70 and Ku86 nuclear expression, as determined by Western blot and immunohistochemical analyses (P<0.001). Cytoplasmic protein expression was found in pathological samples, but not in normal tissues either from tumor patients or from healthy subjects.

CONCLUSION: Overall, our DNA-binding activity and protein level are consistent with a substantial activation of the NHEJ pathway in colorectal tumors. Since the NHEJ is an error prone mechanism, its abnormal activation can result in chromosomal instability and ultimately lead to tumorigenesis.

Key words: Colorectal cancer; Colon adenoma; DNA-dependent protein kinase; Ku70/80 heterodimer; Mismatch repair; Non-homologous end joining; Double strand break repair; Chromosomal instability

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INTRODUCTION

Colorectal cancer is a significant cause of morbidity and mortality in Western populations[1]. This cancer progresses through a series of defined histopathological stages, going from a small benign tumor (adenomatous polyps) to a malignant cancer (carcinoma)[2]. During tumor progression, a stepwise accumulation of genetic changes is observed, leading to inactivation of tumor suppressor genes (e.g. APC, p53) and activation of oncogenes (e.g. K-ras, β-catenin)[2]. The number of genomic alterations in cancer appears to exceed the level possibly due to the accumulation of mutations in cells with normal mutation rates. A number of intricate networks have evolved in eukaryotic cells to respond to exogenous and endogenous genotoxic stimuli[3]. Genes involved in these pathways play

a crucial role in maintaining DNA integrity and a defect in these processes may result in hypersensitivity to DNA damaging agents and genomic instability[4]. Two main forms of genetic instability are associated with tumors. One arises from the inactivation of DNA mismatch repair (MMR) genes[5], leading to instability at the nucleotide sequence level (microsatellite instability, MSI). The other results from a disruption of the pathways intending to protect the cells from chromosomal breakage (double-strand breaks, DSBs), which leads to gross chromosomal rearrangements (chromosomal instability, CIN)[6]. Of the many types of DNA damage, DSBs are the most dangerous, because of the intrinsic difficulty of their repair as compared to other types of DNA damage[6]. In physiological conditions, DNA-DSBs are generated by homologous recombination (HR) during meiosis and occur in other events, such as V(D)J recombination and immunoglobulin class switch[6]. In addition, DSBs can result from both exogenous agents such as ionizing radiation or chemotherapeutic agents and endogenously generated reactive oxygen species[6]. Erroneous rejoining of the broken DNA-DSBs may cause loss or amplification of chromosomal material and even translocations, ultimately leading to tumorigenesis[4].

There are two distinct and complementary mechanisms for DNA-DSB repair: the NHEJ and the HR[4]. Recently, a caretaker role in preventing carcinogenesis has been proposed for the NHEJ pathway[7-9]. At present, five proteins involved in the NHEJ pathway have been identified; namely, the ligase IV and its associated protein XRCC1, and the three components of the DNA-dependent protein kinase (DNA-PK) complex, Ku70, Ku86, and the catalytic subunit PKcs[10]. Mutational analysis has shown that activation of the DNA-PK well correlates with Ku protein heterodimerization and DNA-end binding[11]. Once anchored to the DNA, the Ku70/80 heterodimer translocates along the molecule and facilitates recruitment of the catalytic subunit to the site of the break, to form an activated DNA-PK complex[11]. Numerous studies have investigated the role of MMR pathway in colorectal carcinogenesis, but little is known about the involvement of the DSB repair pathway in the adenoma/carcinoma sequence[11-13]. In the attempt to better understand this role, we analyzed the DNA-binding activity of the Ku70/80 heterodimer and the protein expression of the two Ku subunits in colon adenomas, colorectal cancers and matched normal tissues. Ku70/80 DNA binding activity was increased in approximately 50% of adenoma and carcinoma samples, as compared to matched normal tissues. In tumors with increased DNA-binding activity, Ku70 and Ku86 protein expression correlated with the heterodimer binding activity.

MATERIALS AND METHODS

Patients characteristics and tissue samples

Twenty patients with colon adenoma and 15 patients with colorectal carcinoma were recruited in the study. The patients underwent endoscopic polypectomy or surgical resection between 1999 and 2002, at the Departments of Gastroenterology and General Surgery, Campus Bio-Medico University of Rome, Italy. None of the patients were affected by familial polyposis or HNPCC. Subjects included 20 males (57%) and 15 females (43%), with a mean age of 69.5±12 years (range 35-82 years). None had pre-operative chemotherapy or irradiation. All the patients gave informed consent for the study. Thirty-one lesions (89% including adenomas and carcinomas) were located in the colon and 4 (11%) in the rectum. Adenomas were classified according to the National Polyp Study Cohort and WHO recommendations on the basis of size and grade of dysplasia[14,15]. Clinical staging of colorectal cancer was assessed according to the Dukes' classification[13].

Preparation of cell and nuclear extracts

Following surgical resection, tissue samples were immediately frozen at -80 °C. For each case, before protein extraction, one 3-μm hematoxylin-eosin stained slide was analyzed to ensure that tumor samples contained at least 70% cancer cells. Protein extraction was performed as previously described[16-18]. Briefly, frozen samples were mechanically fractionated to obtain a cellular suspension. Nuclear and cytoplasmic fractions were separated by centrifugation at 10 000 *g* and stored at -80 °C.

Protein content in nuclear and cytoplasmic extracts was determined in triplicate by Bradford assay (Bio-Rad Protein Assay, Bio-Rad Laboratories, Munchen).

Gel-shift assay

Electrophoretic mobility shift assay (EMSA) was performed as described previously[18,19]. Briefly, DNA binding reactions contained 50 000 cpm of the labeled probe, nuclear (2 μg) or cytoplasmic (5 μg) extracts with closed circular plasmid DNA pUC-19 (1 μg) as the unspecific competitor. For each sample, three single shift assays were performed. As controls, 33 normal human tissues from patients without colon tumor were analyzed (mammary gland $n = 8$, bladder mucosa $n = 8$, and skin $n = 17$).

To normalize all the samples, electrophoretic mobility shift assays were performed by incubating the nuclear extracts (3 μg) with 50 000 cpm/sample of ^{32}P-end labeled Sp-1 oligonucleotide (Promega Corporation, Madison, WI, USA) in a binding buffer, with 1 μg of poly (dI-dC) as the unspecific competitor. The correction factor (CF) was calculated as follows: *SP1 binding activity in the sample/mean SP1 binding activity*. Data were normalized using the following formula: *Mean Ku70/80 binding activity/CF*[20].

For gel supershift experiments, goat polyclonal anti-Ku70 and anti-Ku86 antibodies (M-19, M-20: Santa Cruz Biotechnologies Inc., CA, USA) were incubated with protein extracts for 30 min at room temperature, before the other components were added to the binding reaction. Complexes were separated on 6% non-denaturing polyacrylamide gels and exposed to X-ray films (Amersham-Pharmacia Biotech, England HP7 9NA).

The optical densities (OD) were obtained by scanning densitometry using colon carcinoma cell line CaCo-2 (ATCC) as internal control (OD = 10.7±6.4).

Supershift control experiments confirmed the specificity of the results in each gelshift assay experiment.

Immunohistochemistry

Paraffin sections from matched normal colonic mucosa were available for all the colon cancer cases entered in the study, whereas paraffin embedded blocks from matched normal tissues were available only for 10 adenomas. Paraffin sections from five subjects who showed normal colonic mucosa at colonoscopy were also analyzed. Consecutive two micron sections were immunostained for Ku70 and Ku86 following the streptavidin-biotin method, as described previously[20] . In brief, sections were deparaffinized, rehydrated in decreasing alcohol and microwave treated. Endogenous peroxidase activity was quenched by treatment with 0.03% hydrogen peroxide in absolute methanol for 30 min at room temperature. The primary antibodies were goat polyclonal anti-Ku70 and anti-Ku86 antibodies specifically validated for immunohistochemical analysis of paraffin embedded tissues (M-19, M-20: Santa Cruz Biotechnology Inc., CA, USA), in a 1:200 dilution. Biotinylated swine antigoat/mouse/rabbit IgG (Dako A/S, Denmark) was used as secondary antibody. After washing, sections were treated with streptavidin-peroxidase reagent (Dakopatts A\S, Denmark), incubated with diaminobenzidine (DAB) and counterstained with hematoxylin. Slides were examined under a two-head microscope by two pathologists, unaware of the clinical data and molecular results. No discrepant results were identified. Results of the nuclear immunostaining were expressed as percentage of positively stained cells. For cytoplasmic staining the microscopic analysis was not able to discriminate one cell from another. Thus the immunoreactivity was classified into four staining levels (SL): 0 SL (no staining); 1 SL (1-33% of positively stained area); 2 SL (33-66% of the area), and 3 SL (66-99% of the area)[21].

Western blotting

Protein extracts (10 μg) were separated in 10% SDS-PAGE, transferred to a PVDF membrane (Hybond-P, Amersham-Pharmacia Biotech, UK HP7 9NA) using an electroblotting apparatus, and incubated for 1 h at room temperature in 1% BSA, 1% skim milk (Difco Lab., Detroit, MI, USA), and 0.5% Tween 20 (USB, Cleveland, OH, USA). Membranes were stained with Ponceau S dye, to check for equal loading and homogeneous transfer. Immunodetection experiments were performed as described previously[17]. Filters were reprobed with anti β-actin (Sigma-Aldrich, St. Louis, MO 63103, USA) mouse IgG_1 monoclonal antibody, to normalize the nuclear protein levels. Filters were washed and developed using an enhanced chemiluminescence system (ECL, Amersham-Pharmacia Biotech, UK HP7 9NA). The optical densities (OD) were obtained by scanning densitometric analysis of the bands normalized for the β-actin levels, as reference protein.

Statistical analysis

All values provided in the text and figures are means of three independent experiments±standard deviations (SD). Variation rates (VR) were defined by the following formula: [(*pathological sample value–normal sample value)/pathological sample value*]×100. Mean values were compared using the one- or two-tailed Student's *t*-test, for independent samples. $P<0.05$ was considered statistically significant.

RESULTS

Nuclear and cytoplasmic Ku70/80 DNA-binding activity

EMSA were performed on nuclear extracts of colon tissues, to compare the DNA binding activity of the Ku70/80 heterodimer in adenoma and carcinoma samples as well as matched normal colonic mucosa samples (Figure 1A). We found that adenoma and carcinoma samples had an overall increase in DNA binding activity as compared to matched normal samples ($P<0.01$, Table 1). However, these variations were not homogeneous among the tumors that were tested. Since the overall increase in binding activity compared to normal was approximately 50% (Table 1), we set this value as the cut-off for better classifying our pathological samples. Of the 20 adenomas, 11 (55%) showed an increase in DNA binding activity higher than 50% ($P = 0.000$) (Figure 1A, Group I), 6 (30%) displayed an increase in DNA binding activity lower than 50% (P = NS) (Figure 2A, Group II), and 3 (15%) showed reduced levels of activity ($P = 0.04$) (Figure 2A, Group III). Of the 15 carcinomas, 7 (47%) displayed an increase in DNA binding activity higher than 50% (Figure 2B, Group I), 6 (40%) showed an increase in DNA binding activity lower than 50% ($P<0.02$) (Figure 2B, Group II), and 2 (13%) showed reduced levels of activity (P = ND) (Figure 2B, Group III).

No significant differences were found when the Ku70/80 DNA-binding activity was compared in adenoma and carcinoma samples (Table 1). We also determined, if functional Ku70/80 heterodimer was present in cytoplasmic protein extracts. Cytoplasmic Ku70/80 DNA binding activity was only found in three colorectal cancers, whereas adenomas did not show cytoplasmic activity (Figure 1B). No statistically significant correlation was found when results of nuclear and cytoplasmic Ku70/80 DNA-binding activity were compared with clinical parameters.

Table 1 Statistical summary of Ku70/80 DNA binding activity and Ku70 protein expression in pathological samples and matched normal tissues (mean±SD)

Colon	Ku70/80 DNA binding activity			Ku70 protein expression		
Tissue	Normal	Tumor	*P*	Normal	Tumor	*P*
Adenomas	2.66±2.32	5.01±3.15	<0.01	0.88±0.59	1.28±0.45	<0.01
Carcinomas	2.99±2.84	5.35±2.75	<0.001	1.09±0.80	1.77±0.83	<0.01

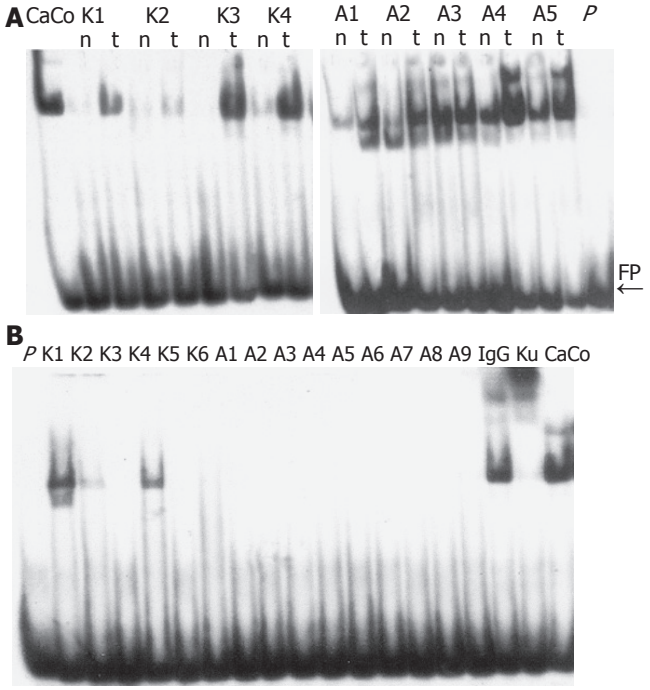

Figure 1 Representative gel shift experiments on nuclear (**A**) and cytoplasmic (**B**) extracts from human colon tissues. Panel A: Normal (n) and pathologic (t) nuclear extracts, obtained from four colorectal cancer (K1-K4) and five adenoma (A1-A5) patients, were analyzed by EMSA as described in "Materials and methods". The positions of Ku band-shifts and of the free probe (FP) are indicated. The binding activity in nuclear extracts from the CaCo-2 cell line (CaCo) has been used as a standard in order to compare the activity in the different gels. Lane P: free probe without protein extract. Panel B: EMSA was performed on pathologic cytoplasmic extracts from colorectal cancer (K1-K6) and adenoma (A1-A9) patients. Lane P: free probe without protein extract.

Ku70 and Ku86 expression in nuclear and cytoplasmic protein extracts

In adenomas and carcinomas with increased DNA-binding activity, the Ku70 nuclear levels were markedly increased as compared to matched normal samples ($P<0.000$) (Figures 3A and B, Group I), whereas protein level changes were not statistically significant in tumors with minimal increases or reduced binding activity (Figures 3A and B, Groups II and III). Representative results are shown in Figure 4.

In all the samples, Ku86 protein levels were always lower than Ku70 and in most cases it was undetectable (data not shown). Analysis of cytoplasmic protein extracts showed barely detectable Ku70 protein in normal colon tissues and a significant increase in pathologic samples with increased DNA binding activity (0.22±0.3 OD and 0.80±0.2 OD, respectively, $P = 0.001$) (data not shown). Ku86 protein subunit was undetectable by Western blot analysis on cytoplasmic extracts.

Ku70 and Ku86 immunohistochemical analysis

A significant increase in the percentage of stained nuclei was found for Ku70 and Ku86 subunits in all adenoma and carcinoma samples, as compared to matched normal tissues ($P<0.000$) but no statistically significant differences were found between adenoma and carcinoma samples (Table 3). In all cases, Ku subunits were not detected in the cytoplasm of normal colonic mucosa ($n = 25$) from patients or healthy donors ($n = 5$) (SL 0). For Ku70, a low level (SL 1) of cytoplasmic staining was detected in 11 of the 20 adenomas (55%), and in 3 of the 15 carcinomas

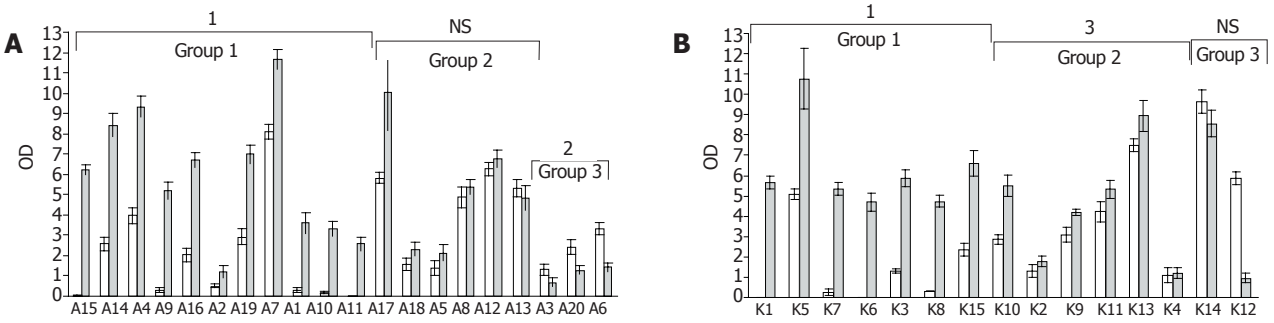

Figure 2 Changes in Ku70/Ku80 heterodimer DNA binding activity in adenoma (**A**) and carcinoma (**B**) compared to matched normal colonic mucosa. □ Normal; ▨ Tumor; vertical bars indicate standard deviation from the mean of three independent experiments. Adenomas (**A**) and carcinomas (**B**) samples characterize by: Group 1 more than 50% increase in DNA binding activity; Group 2 modest increase in binding activity; Group 3 reduced binding activity, $^1P<0.000$ *vs* group 1; $^2P<0.04$ *vs* group 3; $^3P<0.02$ *vs* group 2.

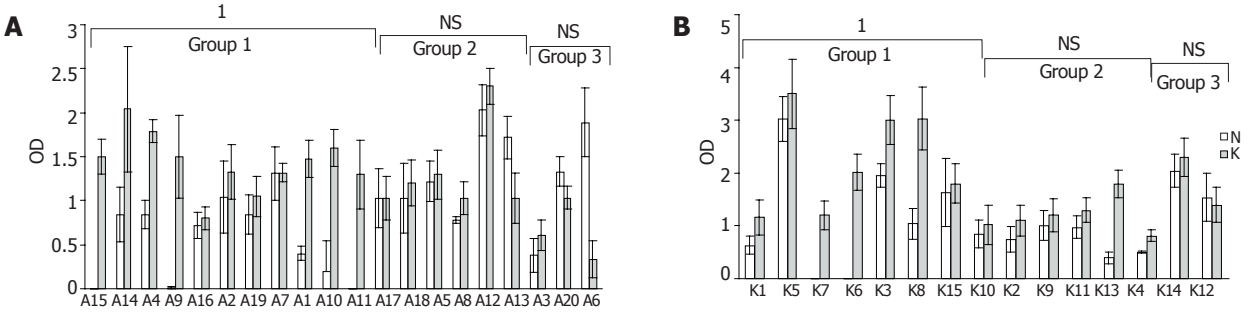

Figure 3 Changes in Ku70 protein levels determined by Western blot analysis in adenoma (**A**) and carcinoma (**B**) compared to matched normal colonic mucosa. □ Normal; ▨ Tumor; vertical bars indicate standard deviation from the mean of three independent experiments. Adenomas (**A**) and carcinomas (**B**) are grouped according to the criteria described in Figure 2, $^1P<0.000$ *vs* group 1.

(20%, $P = 0.089$). For Ku86, 17 of the 20 adenomas (85%) showed medium levels of cytoplasmic staining (SL 2-3), and 5 of the 15 carcinomas (34%) displayed cytoplasmic staining with SL 1-2 ($P = 0.004$). Ku86 cytoplasmic staining showed a typical granular pattern, probably due to the presence of protein subunit in cytoplasmic vesicles (Figure 5).

DISCUSSION

The NHEJ pathway plays a pivotal role in the repair of DSBs. Several studies on cell lines and knock-out mice suggest that a non-functional NHEJ can induce an increased level of genomic instability and lead to cancer progression[22-24]. In a previous study, we found a downregulation of the Ku70/80 DNA binding activity in advanced breast and bladder human cancers, as compared to non-invasive or low stage tumors[17]. This observation was also confirmed in a patient affected by sporadic multiple basal cell carcinoma, where we demonstrated a differential modulation of the NHEJ pathway in non-aggressive and aggressive tumors, showing that the first is an upregulation system, and the latter a strong downregulation system[20].

In the present study, we investigated the involvement of the ku70/80 heterodimer during progression from adenoma to carcinoma. Our results indicated that the NHEJ pathway was activated in approximately half of the cases, with no differences in binding activity between adenomas and carcinomas. In these tumors, the increase in DNA binding activity correlated with protein levels of the Ku subunits as determined by Western blot and immunohistochemical analysis. Moreover the cytoplasmic accumulation of the Ku70 and Ku86 protein subunits only in neoplastic tissues is also indicative of an upregulation of the NHEJ system. The NHEJ pathway repairs DSBs by modifying the two DNA broken ends prior to rejoining, and few nucleotides at each end of the DNA break are lost during this process[23]. Thus, the repair through the NHEJ can potentially lead to chromosomal alterations. Rothkamm *et al*.[25] found that after X-ray exposure, NHEJ-proficient cells form misrejoinings and multiple DSBs more frequently than NHEJ deficient cells. Also, myeloid leukemia cells characterized by gross chromosomal abnormalities show a higher end-joining efficiency but a lower DSB repair fidelity, as compared to lymphocytes from healthy donors[24]. Thus, the upregulation of the NHEJ system in adenoma and colon carcinoma may be

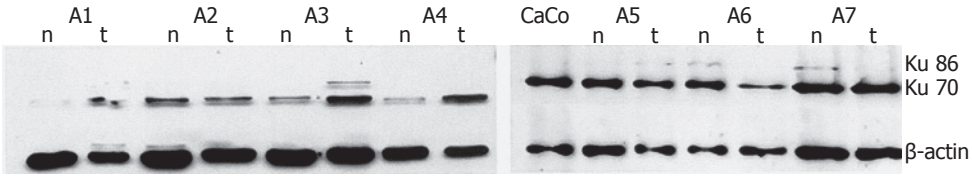

Figure 4 Representative immunoblot experiments on nuclear extracts from adenoma cases p70 and p86 protein expression was evaluated by Western blot, on normal (n) and pathologic (t) nuclear extracts from adenoma patients (A1-A7). The p86 protein was slightly detectable on this and on the other filters analyzed. CaCo-2 cell line nuclear extracts (CaCo) were used as internal standard. Filters were reprobed with anti-β-actin monoclonal antibody to normalize the protein levels.

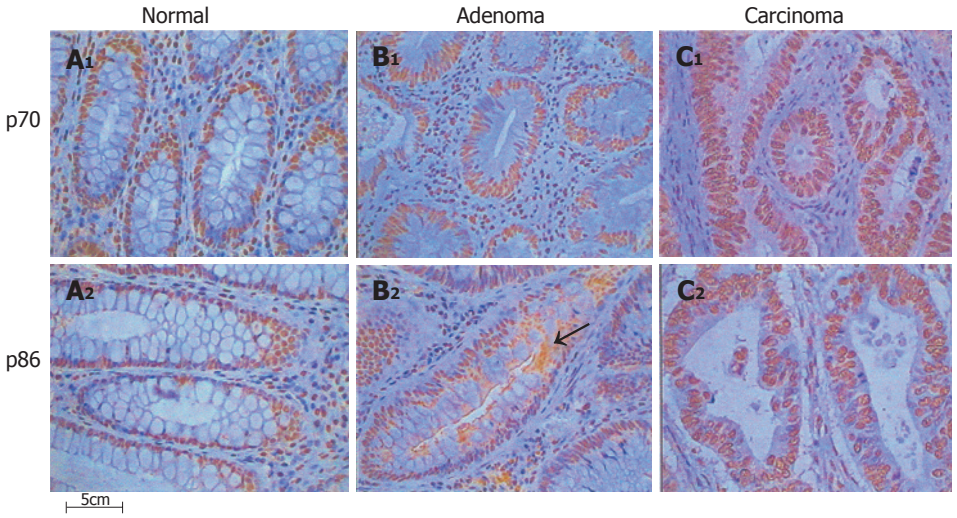

Figure 5 Expression of DNA-PK protein subunits in human colon tissues. Tissue sections were stained by immunohistochemistry for Ku70 and Ku86 proteins, as described in "Materials and methods". The increase of positively stained cells is evident in the nuclei of adenoma and carcinoma sections, respect to the normal controls. Ku70 nuclear expression is uniform and displays a higher intensity of staining as compared with p86, as described in the text. No cytoplasmic expression is evident in the normal mucosa sections for any of the protein subunits, whereas it is clearly visible in the pathologic tissues. Ku86 was expressed in the cytoplasm following a speckled pattern of staining, as evident on the adenoma section (arrow). Original magnification ×400. **A**$_{1\text{-}2}$: Normal; **B**$_{1\text{-}2}$: Adenoma; **C**$_{1\text{-}2}$: Carcinoma.

Table 2 Ku70 and Ku86 nuclear immunostaining in adenomas and matched normal tissues

Adenomas	Ku70		Ku86	
	Normal	Tumor	Normal	Tumor
A1	61	93	40	81
A2	65	81	33	70
A3	60	83	31	63
A4	61	82	35	71
A5	65	79	32	75
A6	58	68	39	62
A7	64	74	26	62
A16	51	75	25	71
A17	79	92	24	63
A19	61	87	29	62
Carcinomas				
K1	66	95	27	78
K2	48	76	37	62
K3	66	84	26	71
K4	47	86	40	86
K5	72	85	34	70
K6	76	86	26	61
K7	72	88	28	76
K8	51	92	17	79
K9	62	96	44	65
K10	65	90	51	71
K11	56	94	51	66
K12	52	82	34	73
K13	65	85	25	66
K14	52	92	18	64
K15	65	73	28	65

responsible for an increase in genomic rearrangements and chromosomal defects, contributing to tumor progression.

Although increase in DNA binding activity was the more frequent abnormality detected in our series of adenomas and carcinomas, we also identified a subset of colon neoplasia (approximately 15%) that showed reduced levels of DNA binding activity. Rigas *et al*.[26] have previously analyzed protein expression of the DNA-PK subunits in adenomas and colorectal cancers by IHC assay. By using a score that correlates the intensity of staining to the percentage of stained cells, a decrease in protein expression levels is demonstrated for all DNA-PK subunits in adenoma and carcinoma samples, as compared to normal tissues[26]. In our study the reduction in DNA binding activity did not correlate completely with Ku subunit protein levels. While three of the adenomas and one of the cancers showed a decrease of Ku70 expression by Western blot analysis, the remaining adenoma and carcinoma samples displayed an increase in protein expression. IHC data also showed an increase in the percentage of nuclear stained cells in the tumor as compared to the normal colonic mucosa. We can speculate that in this subset of tumors the NHEJ proteins are expressed and localized in the nuclei, but they are not functional, due to post-translational regulation mechanisms or mutations in one or more components of the NHEJ pathway.

Overall, our results indicate that the NHEJ pathway

Table 3 Statistical summary of Ku70 and Ku86 nuclear immunostaining in pathological samples and matched normal tissues (mean±SD)

Colon tissue	Ku70			Ku86		
	Normal	Tumor	*P*	Normal	Tumor	*P*
Adenomas	63.39±7.72	80.22±7.52	0.000	30.48±5.43	67.57±7.64	0.000
Carcinomas	61.42±9.66	87.34±6.97	0.000	32.86±11.29	70.36±7.55	0.000
Healthy	74.20±6.38	-	-	32.00±5.10	-	-

is activated in colon adenoma and carcinoma, with only a subset of tumors showing decreased binding activity. These results however do not exclude an involvement of the NHEJ pathway in colon carcinogenesis, rather suggest the presence of colon cancer subsets that may differ in their biological behavior. Since Ku70 and Ku80 protein levels are correlated to tumor radiosensitivity and response to chemotherapy in human colorectal cancer and experimental models[27–30], the clarification of the mechanisms involved in DNA repair may ultimately lead to an improved management of colorectal cancer patients.

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• *BRIEF REPORTS* •

Correlation and prognostic significance of beta-galactoside alpha-2,6-sialyltransferase and serum monosialylated alpha-fetoprotein in hepatocellular carcinoma

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Abstract

AIM: To investigate the correlation between tissue ST6Gal I and serum msAFP in HCC patients, and to investigate their prognostic significance.

METHODS: Preoperative sera, paired tumorous and non-tumorous tissues were collected from 19 consecutive patients who had undergone surgical resection of HCC. ST6Gal I activities in the tissues were measured by an *in vitro* microsomal enzyme activity assay. The percentages of tumor-specific msAFP in the sera were also estimated by an isoelectric focusing-immunoblotting assay.

RESULTS: The tumor ST6Gal I activity was negatively correlated with serum msAFP percentage (r = -0.53, P = 0.019). Both decreased tumor ST6Gal I activity and increased serum msAFP percentage were associated with poor tumor cell differentiation. Univariate analyses showed that both decreased tumor ST6Gal I activity (P = 0.028), increased serum msAFP percentage (P = 0.034) and poor tumor cell differentiation (P = 0.031) were associated with shorter overall survival. Multivariate analysis using the Cox regression model showed that the preoperative serum msAFP percentage (P = 0.022) and tumor cell differentiation status (P = 0.048) were independent prognostic indicators for patient overall survival.

CONCLUSION: Our results indicate that the presence of msAFP in blood circulation is associated with a decreased activity of ST6Gal I activity in HCC. Both tissue ST6Gal I and serum msAFP are potential prognostic markers for patients with operable HCC.

Key words: Alpha-fetoprotein; Beta-galactoside alpha-2,6-sialyltransferase; Hepatocellular carcinoma; Patient survival; Cell differentiation

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INTRODUCTION

Beta-galactoside alpha-2,6-sialyltransferase (ST6Gal I) is the key enzyme for the production of alpha-2,6-linked sialoglycoconjugates. Both ST6Gal I and alpha-2,6-linked sialoglycoconjugates have been suggested to play important roles in oncogenic transformation and metastasis[1-3]. Upregulated expression of ST6Gal I has been shown in colorectal cancer[4,5], breast cancer[6], cervical cancer[7], and choriocarcinoma[8]. However, elevated alpha-2,6 sialylation inhibited formation of glioma *in vivo*[9]. Expression of ST6Gal I may have different effects in different cancer types.

Serum alpha-fetoprotein (AFP) is a conventional marker for the diagnosis of hepatocellular carcinoma. Eighty to ninety percent of patients with HCC will have levels above the reference range[10-12]. A serum concentration >500 μg/L, in an area with high incidence of HCC,

and in the appropriate clinical setting, is usually considered diagnostic of HCC. However, modestly raised levels of AFP (10-500 μg/L) are also common in non-malignant chronic liver disease, so that the specificity of the AFP test for HCC tends to be low[10,13-15]. Recent studies have shown that monosialylated AFP (msAFP), which is a hyposialylated isoform of alpha-fetoprotein (AFP), is specific to hepatocellular carcinoma (HCC)[16,17]. msAFP can be identified, measured quantitatively by isoelectrofocusing electrophoresis approach or by glycosylation immunosorbent assay[18]. msAFP percentage (msAFP%) relative to total AFP can be used as a serum marker to differentiate HCC patients with non-diagnostic total AFP from patients with chronic liver diseases[18].

Sialylation of AFP is mediated by cellular ST6Gal I. We hypothesize that downregulation of ST6Gal I plays an important role in the HCC pathogenesis, and causes the presence of msAFP in blood circulation of HCC patients. In the present study, we examined the enzyme activity and mRNA expression of ST6Gal I in the paired tumorous and non-tumorous tissues from patients with HCC. The correlation among ST6Gal I activity, serum msAFP percentage and their clinical implications were investigated.

MATERIALS AND METHODS

Clinical materials

Between December 1999 and January 2001, 19 consecutive patients who had undergone surgical resection of HCC, which contained viable tumor cells as shown by histological examination, and with serum AFP levels higher than 20 ng/mL were recruited into this study. Informed consent was obtained for using the resected tissues and sera for research studies. The patients were followed up for 2 years or until death. Preoperative sera, paired tumorous and non-tumorous tissues were collected. Sera were stored at -20 °C until further analysis. The tissues were cut into small pieces, snap frozen with liquid nitrogen, and stored at -70 °C. The AFP levels in the serum samples were quantified with a commercial sandwich-type ELISA (DAKO, Glostrup, Denmark). This study was approved by the Joint CUHK-NTEC Clinical Research Ethics Committee.

Preparation of tissue microsomal fraction

The tissues were homogenized on ice in 1 mL of sodium phosphate buffer (PBS, 10 mmol/L $NaPO_4$, 150 mmol/L NaCl, pH 7.4), containing 0.8 mmol/L protease inhibitor (Pefabloc® SC PLUS, Boehringer Mannheim, Roche Diagnostics, Germany), with a Polytron-Aggregate (Kinetica, Switzerland) using a 11-mm cutting probe at a speed setting of 5 for 15 s. After centrifugation at 4 °C for 10 min at 1 000 r/min, the supernatant fraction was saved and centrifuged again at 13 000 *g* for 30 min at 4 °C. The microsomal pellet obtained after centrifugation was then resuspended in Tris-HCl buffer (15 mmol/L Tris-HCl, pH 6.0) and stored at -70 °C until analyzed. The protein content was measured by the Bradford Coomassie Dye-Binding Protein Assay.

ST6Gal I activity assay

The assay was similar to the method described by Pousset *et al*. and Halliday *et al*. It was based on the specific binding property of *Sambucus nigra agglutinin* (SNA, Calibiochem, San Diego, USA) that preferentially binds sialic acid residues attached to terminal Galβ1, 4GlcNAc units on *N*-glycan in alpha-2,6, but not 2,3 or 2,8 linkage[19,20].

Briefly, the microsomal preparation was used as a source of alpha-2,6-sialyltransferase, and the *N*-glycan on asialofetuin (ASF) (Sigma, St. Louis, USA) was used as a sialic acid acceptor. The ST6Gal I activity assay was carried out in a mixture containing 10 μg of tissue microsome preparation, 125 μg ASF, 125 μmol/L CMP-sialic acid and 0.25 μCi CMP-[^{3}H]-sialic acid in reaction buffer (0.1% BSA, 0.1% Triton X-100, 45 mmol/L NaCl, 15 mmol/L Tris-HCl, pH 6.0) with a final volume of 20 μL. The mixture was added to 30 μL of SNA-sepharose (1 mg SNA covalently linked to 0.3 g of CNBr activated Sepharose 4B gel), mixed, and incubated at 37 °C overnight. After washing the gels four times with washing buffer (PBS containing 0.5% Tween 20), the molecules bound to the SNA-Sepharose were released by vortexing the gel in 300 μL of 1 mol/L H_2SO_4 for 30 s. Two hundred and eighty-five microliters of the supernatant was transferred to a vial, and 5 mL scintillant solution was added. The amount of radiolabeled ASF was then measured with a liquid scintillation counter. Commercial rat ST6Gal I (Sigma) was used as the calibration standard. One unit of the commercial rat ST6Gal I is defined as the amount that will transfer 1.0 μmol of sialic acid from CMP-sialic acid to asialomucin per minute at pH 6.5.

Semi-quantitation of ST6Gal I mRNA by RT-PCR-ELISA

Ten to thirty milligrams of frozen tumorous or non-tumorous liver tissue was disrupted and homogenized using a mortar and pestle (Kontes, Vineland, NJ, USA). Total RNA was extracted from the tissue sample using the RNeasy Mini Kit (Qiagen, GmbH, Germany), and reverse transcribed into cDNA by using a 1st strand cDNA Synthesis Kit (AMV, Roche Diagnostic, Boehringer Mannheim, Germany) with the oligo-$p(dT)_{15}$ primer provided. The cDNA was then used as the template for PCR amplification with primers specific to ST6Gal I (forward: 5'-CCTGAACAATTCCAGCCT GCTCCTTT-3' and reverse: 5'-GACGATGTTTCCA ATCCCCTGTACCA-3') or β-actin gene (forward, 5' -CTTCTACAATGAGCTGCGT-3' and reverse: 5'-TCAT GAGGTAGTCAGTCAG-3'). In the PCR for ST6Gal I, the cDNA, diluted in Tris–EDTA buffer (10 mmol/L Tris-HCl, 1 mmol/L EDTA, pH 8.0), was mixed with 1×PCR buffer, 2 mmol/L DIG-labeled dNTP, 2.5 mmol/L $MgCl_2$, 1 μmol/L of each primer, 0.83 U *Taq* DNA polymerase, 0.022 μmol/L TaqStart Antibody, 0.8 U uracil glycosylase in a final volume of 20 μL. The mixture was incubated at room temperature for 10 min. Then an initial denaturation step of 2 min at 94 °C was done, followed by repeating

cycles of 1 min at 94 °C, 1 min at 63 °C for primer annealing, and 1 min at 72 °C for extension. A final extension run of 7 min at 72 °C was performed to ensure complete elongation of all amplicons. In the PCR for β-actin gene, 3.5 mmol/L of $MgCl_2$ was used instead of 2.5 mmol/L, and the primer annealing temperature in each PCR cycle was 60 °C instead of 63 °C. In the initial attempts, 40 cycles of PCR were performed. The specificity of the PCR amplication was checked by agarose gel electrophoresis to see if the sizes of the amplicons were the same as the expected values, 421 bp for ST6Gal I cDNA and 305 bp for β-actin gene. A commercially available cDNA preparation of human normal liver tissues, pooled from two male/female Caucasians, was used as positive control (Clontech Laboratories, Inc., CA, USA).

For the semi-quantitative assay, 25 cycles of PCR were performed in the presence of DIG-labeled dNTP. The concentrations of the DIG-labeled amplicons were measured with the PCR ELISA (DIG-detection) kit (Roche Diagnostics), according to the manufacturer's instruction. Briefly, the DIG-labeled amplicons were first denatured, and hybridized with 7.5 pmol biotinylated DNA probe specific for the ST6Gal I (biotin-5'-TGCATTGGGC ACAATTGTAA-3') or β-actin gene (biotin-5'-GTCCAGA CGCAGGATGGCAT-3'). The DNA-probe hybrids were then captured onto an avidin-coated microplate. After washing, the amount of the DNA-probe hybrids bound to the microplate was determined by incubating with anti-DIG-HRP conjugate at 37 °C for 30 min, followed by adding the BM Blue peroxidase substrate (Roche Diagnostics). One hundred microliters of 1 mol/L H_2SO_4 was added to stop the colorimetric reaction. The optical density of the wells was determined at 450 nm with a reference wavelength of 690 nm. The ST6Gal I/β-actin mRNA ratios were calculated as the mRNA values of the tissue ST6Gal I.

Semi-quantitation of msAFP percentage by IEF-immunoblotting assay

The semi-quantitative analysis of msAFP was performed as previously reported by us[18]. AFP isoforms in the serum samples were separated by IEF on a polyacrylamide gel, which were pre-swollen with a solution containing 5 mol/L urea and 1:16 Pharmalyte 4.5-5.4 (Amersham Pharmacia Biotech, Uppsala, Sweden) in double distilled water. One microliter of pre-diluted serum samples or standard containing 5 or 10 ng/mL AFP. The total AFP was applied to the anode side of the gel after prefocusing (2 000 V, 2.0 mA, 3.5 W, 10 °C, 75 V.h). The sample was applied for 15 V.h (200 V, 2.0 mA, 3.5 W, 10 °C). The final isoelectric separation step was done for 450 V.h (2 000 V, 5.0 mA, 3.5 W, 10 °C). The focused proteins were transferred to nitrocellulose membrane, and then incubated with polyclonal rabbit anti-human AFP (DAKO), followed by horseradish peroxidase conjugated polyclonal swine anti-rabbit immunoglobulin (DAKO). After washing, the enhanced chemiluminescence detection system (ECL, Amersham Pharmacia Biotech) was used to visualize the AFP protein bands. The image of each band was scanned with a densitometer (GS-700, Bio-Rad, CA, USA), and the intensity of each band was expressed as a percentage of the total intensity of all AFP bands.

Statistical analysis

The Wilcoxon signed rank test was used to compare the differences between the paired tumor and non-tumor groups. The Mann–Whitney rank sum test and Fisher's exact test were used to compare the differences between other study groups. Correlations between the study parameters were analyzed by the Spearman's rank order correlation test. The log-rank test and Cox proportional hazards model were applied for survival analyses.

RESULTS

Two HCC subgroups with increased and decreased tumor ST6Gal I activities

The clinical features of the 19 patients with primary HCC are summarized in Table 1. The tumor ST6Gal I activity was compared to that of the paired non-tumorous tissue. Seven HCC patients fell into a group with decreased ST6Gal I activity in the tumorous tissue, whereas 12 HCC patients fell into a group with increased ST6Gal I activity in the tumorous tissue (Table 2). The tumor ST6Gal I activities in the decreased group were significantly lower than the values in the increased group ($P = 0.005$).

Absence of correlation between ST6Gal I activity and mRNA level in HCC

The specificity of the RT-PCR reaction was checked by subjecting the RT-PCR product to agarose gel electrophoresis. In all cases including the positive control (normal human liver cDNA), the RT-PCR product appeared as a single DNA band with the expected size of 421 bp for ST6Gal I, or 305 bp for β-actin. The electrophoresis results indicated that the RT-PCR amplifications were specific. Our data also confirmed that ST6Gal I gene was expressed in all the HCC tissues and the non-tumorous liver tissues (Figure 1). Two cases were omitted for the RT-PCR ELISA to measure ST6Gal I mRNA level owing to insufficient tissue materials. The measured ST6Gal I mRNA level of individual tissues was normalized by expressing the data as a ratio of β-actin mRNA level. In the non-tumorous tissues, the ST6Gal I mRNA level positively correlated with the ST6Gal I activity ($r = 0.49$, $P = 0.039$). In the tumorous tissues, no significant correlation was found between the enzyme activity ($r = -0.093$, $P = 0.72$).

Correlation between tumor ST6Gal I enzyme activity and serum msAFP

The percentages of tumor-specific msAFP (relative to total intensity of AFP isoforms) in the preoperative sera of the HCC patients were estimated, and the results of the patient groups with increased and decreased tumor ST6Gal I enzyme activity are shown in Table 2. Comparison of the

Table 1 Clinical features of the studied subjects with operable HCC

Case	Sex	Age	HBs Ag positivity	Cirrhosis	Tumor stage		Tumor size	Tumor differentiation	Encapsulation	Capsule invasion	Vascular invasion	Preoperative serum AFP (ng/mL)
					ALTSG	AJCC	(cm)					
1	F	51	Yes	Yes	T2	T2	2.4	Moderate	Yes	Yes	No	20
2	M	75	Yes	Yes	T2	T2	3.4	Moderate	Yes	Yes	No	25
3	M	52	Yes	No	T3	T2	10	Moderate	Yes	No	No	64
4	M	44	Yes	Yes	T3	T3	4	Well	Yes	Yes	Yes	69
5	M	44	No	Yes	T2	T2	2.6	Poor	Yes	Yes	No	73
6	M	53	Yes	Yes	T3	T2	8.3	Poor	Uncertain	Uncertain	No	100
7	M	65	No	Yes	T2	T2	4.4	Moderate	Yes	No	No	150
8	F	41	Yes	Yes	T3	T2	7	Well	Yes	Yes	No	151
9	M	57	No	Yes	T3	T2	6	Poor	Yes	Yes	Yes	152
10	M	46	Yes	Yes	T1	T1	1.2	Moderate	Yes	Yes	No	365
11	M	60	Yes	Yes	T2	T2	2.2	Poor	Yes	Yes	No	618
12	M	67	Yes	Yes	T2	T2	2.5	Moderate	Yes	Yes	No	1 057
13	M	48	Yes	No	T2	T2	4	Moderate	Yes	No	No	1 427
14	M	43	Yes	No	T2	T2	2.5	Moderate	Yes	Yes	No	1 500
15	F	31	Yes	Yes	T2	T2	2	Moderate	Yes	No	No	2 726
16	M	40	Yes	No	T2	T2	1.6	Moderate	No	No	No	3 338
17	M	35	Yes	No	T3	T2	8	Poor	Yes	No	No	5 505
18	M	42	Yes	Yes	T2	T2	3.4	Poor	Yes	Yes	No	12 185
19	M	72	Yes	No	T3	T3	6.5	Poor	Yes	Yes	No	42 837

Table 2 The average values of relative ST6Gal I activity, the relative ST6Gal I mRNA level, serum msAFP percentage in the patients with decreased (group 1) and increased (group 2) tumor ST6Gal I activity compared to that of non-tumorous tissue

	Group 1: decreased ST6Gal (n = 7)	Group 2: increased ST6Gal (n = 12)	P[1]
Tumor ST6Gal I activity (mU/mg of protein)	0.57 (0.47, 0.37-0.59)	1.76 (1.21, 0.94-1.93)	0.005
Relative tumor ST6Gal I activity (against non-tumor ST6Gal I activity)	0.46 (0.42, 0.25-0.71)[2]	2.04 (1.51, 1.35-1.88)	<0.001
Tumor ST6Gal I mRNA level[3]	1.78 (0.64, 0.24-1.7)	1.51 (1.08, 0.68-2.6)	N.S[4]
Serum AFP level (ng/mL)	7687 (680, 246-145045)	739 (161, 72-1 197)	N.S.
Serum msAFP percentage (%)	35 (41, 23-45)	18 (13, 11-17)	0.031

[1]Mann–Whitney test; [2]mean (median, 25th-75th percentile); [3]group 1: n = 6, group 2: n = 11; [4]N.S., not significant.

two groups shows that the percentage of msAFP in the patient group with decreased tumor enzyme activity was significantly higher than those in the group with increased activity (P = 0.031), whereas the serum total AFP levels were not significantly different. Furthermore, the msAFP percentage was negatively correlated with the relative tumor ST6Gal I enzyme activity (r = -0.53, P = 0.019).

Associations with poorly differentiated HCC

The relationships between ST6Gal I activity, msAFP percentage and various patient clinical parameters, including HBV status, tumor stage (ALTSG and AJCC), tumor size, differentiation status, vascular invasion, encapsulation, capsule invasion, metastasis and recurrent, were investigated. More cases with poorly differentiated tumor were found in the patient groups with decreased enzyme activity (P = 0.045, Figure 2). Similar to tumor ST6Gal I activity, but in an opposite manner, more cases with poorly differentiated tumor were found in the patient group with higher (>20%) msAFP percentage (P = 0.045, Figure 2).

Associations with poor overall survival

During the 2 years follow-up period, 8 out of the 19 patients died. The absolute/relative tumor ST6Gal I activity, serum msAFP percentage, serum AFP level and various patient clinical parameters were subjected to survival analyses. In univariate analyses using the Cox regression model, the preoperative serum msAFP percentage (P = 0.034), the relative tumor ST6Gal I activity (P = 0.028) and the tumor differentiation status (P = 0.031) were shown to be predictors for patient overall survival. The log-rank test results showed that the patient group with decreased tumor ST6Gal I activity had an overall survival (P = 0.0099, Figure 3A) shorter than the group with increased activity. When the cases were divided into two groups based on the preoperative serum msAFP percentage at a cut-off value of 20%, the log-rank test results showed that higher preoperative serum msAFP percentage was associated with poorer patient overall survival (P = 0.0031, Figure 3B). The log-rank test results also showed that patients with poorly differentiated tumor had poorer overall survival (P = 0.0063). In a multivariate analysis using the Cox regression model, among all the

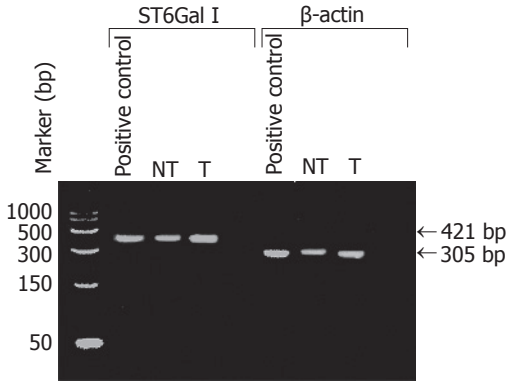

Figure 1 Electrophoresis of RT-PCR products amplified from the total RNA preparations of the tumor (T) and non-tumor liver (NT) tissues from HCC patients. For both tumor and non-tumor total RNA preparations, the RT-PCR products corresponding to ST6Gal I and β-Actin DNA showing the expected sizes (421 bp and 305 bp respectively) were obtained. Commercially available human normal liver cDNA was used as the positive control. Similar DIG-labeled RT-PCR products with the same sizes were obtained when DIG-dNTPs were used.

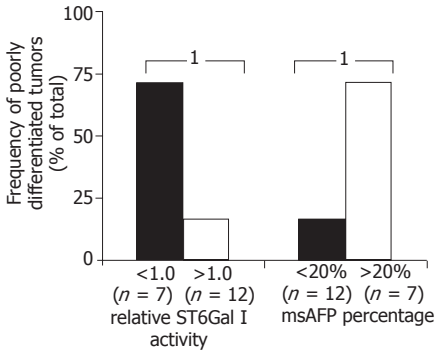

Figure 2 The frequency of poorly differentiated tumors among the 19 HCC patients with different relative tumor ST6Gal I activity or with different preoperative serum msAFP percentage. Differences between the study groups were tested by Fisher's exact test. $^1P = 0.045$.

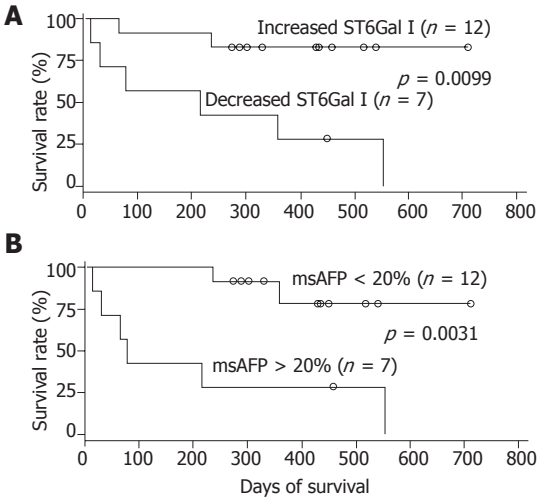

Figure 3 Survival curves of 19 patients with operable hepatocellular carcinoma after surgical resection according to relative tumor ST6Gal I activity (**A**) and preoperative serum msAFP percentage (**B**). Censored cases (○).

clinical features, only the preoperative serum msAFP percentage ($P = 0.022$) and tumor cell differentiation status ($P = 0.048$) were found to be independent prognostic indicators for patient overall survival.

DISCUSSION

Both ST6Gal I and alpha-2,6-linked sialoglycoconjugates play important roles in oncogenic transformation and metastasis in various cancers[21]. However, information about the influence of ST6Gal I in human HCC tissue has been very limited. The results of the present study were consistent to the findings reported by Cao *et al*. and Dall' Olio *et al*.[22,23]. Dalli'Olio *et al*. observed that both ST6Gal I activity can undergo up- or down-regulation in different HCC patients. The present study confirmed Dalli'Olio *et al*.'s observation. Compared to non-tumorous tissue, the HCC patients could be divided into two groups with increased and decreased ST6Gal I activity in the tumorous tissue. By immunohistochemical staining, Cao *et al*. showed that expression levels of ST6Gal were decreased in poorly differentiated HCC. This is also consistent to our observation that poorly differentiated HCCs had lower ST6Gal I activities.

The present study provided additional information about ST6Gal I in HCC. We showed that changes of ST6Gal I activity in HCC positively correlated with the presence of msAFP in blood circulation. As sialylation of AFP is mediated by ST6Gal I, probably the presence of hyposialyled AFP variants in blood circulation is caused by decreased ST6Gal I activity. Furthermore, our data suggests that tissue ST6Gal I and serum msAFP are potential prognostic markers for patients with operable HCC.

In the non-tumorous tissues, ST6Gal I mRNA level positively correlated with ST6Gal I activity. This result is consistent to the observation of Svensson *et al*. when studying normal liver[24]. This result therefore could be served as a positive control to confirm the validity of our measurements of tissue ST6Gal I activity and mRNA level. No significant correlation was found in the tumorous tissues. This suggests that ST6Gal I activity in HCC is regulated at post-transcriptional level.

The present study and the studies from Cao *et al*. and Dall'Olio *et al*. strongly indicate that the functional roles of ST6Gal I in HCC are different from those in colorectal cancer and in breast cancer. An *in vitro* antisense DNA experiment has shown that upregulation of ST6Gal I plays an important role in the invasive potential of human colon carcinoma HT29 cells[3]. Furthermore, high expression levels of ST6Gal I have been correlated with poor survival in colorectal cancer patients[25]. In breast cancers, high ST6Gal I expression was associated with histoprognostic grade III, and negatively correlated to progesterone receptor expression[26]. However, no ST6Gal I expression was found in malignant gliomas or in medulloblastomas[26]. It is worth noting that elevated alpha-2,6 sialylation

inhibited formation of glioma *in vivo*[9]. All these findings indicate that expression of ST6Gal may have different effects in different cancer types.

In conclusion, HCC can be divided into two subtypes, one with decreased tumor ST6Gal I activity and one with increased tumor ST6Gal I activity. The ST6Gal I activity in HCC is not regulated at the transcription level. Downregulation of tumor ST6Gal I activity and an increase in msAFP percentage in preoperative serum are associated with poorly differentiated tumors and poor patient survival. Both ST6Gal I and msAFP percentage are potential prognostic markers for HCC. The presence of msAFP in the blood circulation probably reflects the downregulation of ST6Gal I activity in the HCC tissue.

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• *BRIEF REPORTS* •

Cysteamine increases expression and activity of H^+-K^+-ATPase of gastric mucosal cells in weaning piglets

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Abstract

AIM: To determine the *in vivo* and *in vivo* effects of cysteamine (CS) on expression and activity of H^+-K^+-ATPase of gastric mucosal cells in weaning piglets.

METHODS: Eighteen litters of newborn Xinhuai piglets were employed in the *in vivo* experiment and allocated to control and treatment groups. From 12 d of age (D12), piglets in control group were fed basal diet, while the treatment group received basal diet supplemented with 120 mg/kg CS. Piglets were weaned on D35 in both groups. Six piglets from each group (n = 6) were slaughtered on D28 (one week before weaning), D35 (weaning), D36.5, D38, D42, and D45 (36 h, 72 h, one week and 10 d after weaning), respectively. Semi-quantitative RT-PCR was performed to determine the levels of H^+-K^+-ATPase mRNA in gastric mucosa. H^+-K^+-ATPase activity in gastric mucosa homogenate was also determined. Gastric mucosal epithelial cells from piglets through primary cultures were used to further elucidate the effect of CS on expression and activity of H^+-K^+-ATPase *in vivo*. Cells were treated for 20 h with 0.001, 0.01, and 0.1 mg/mL of CS (n = 4), respectively. The mRNA expression of H^+-K^+-ATPase and somatostatin (SS) as well as the H^+-K^+-ATPase activity were determined.

RESULTS: *in vivo*, both mRNA expression and activity of H^+-K^+-ATPase in gastric mucosa of control group exhibited a trend to increase from D28 to D45, reaching a peak on D45, but did not show significant age differences. Furthermore, neither the mRNA expression nor the activity of H^+-K^+-ATPase was affected significantly by weaning. CS increased the mRNA expression of H^+-K^+-ATPase by 73%, 53%, 30% and 39% on D28 (P = 0.014), D35 (P = 0.017), D42 (P = 0.013) and D45 (P = 0.046), respectively. In accordance with the mRNA expression, H^+-K^+-ATPase activities were significantly higher in treatment group than in control group on D35 (P = 0.043) and D45 (P = 0.040). *In vivo*, CS exhibited a dose-dependent effect on mRNA expression and activity of H^+-K^+-ATPase. Both H^+-K^+-ATPase mRNA expression and activity in gastric mucosal epithelial cells were significantly elevated after 20 h of exposure to the moderate (H^+-K^+-ATPase expression: P=0.03; H^+-K^+-ATPase activity: P = 0.014) and high concentrations (H^+-K^+-ATPase expression: P=0.017; H^+-K^+-ATPase activity: P = 0.022) of CS. Significant increases in SS mRNA expression were observed to accompany the elevation of H^+-K^+-ATPase expression and activity induced by the moderate (P = 0.024) and high concentrations (P = 0.022) of CS. Low concentration of CS exerted no effects either on expression and activity of H^+-K^+-ATPase or on SS mRNA expression in cultured gastric mucosal epithelial cells.

CONCLUSION: No significant changes are observed in mRNA expression and activity of H^+-K^+-ATPase in gastric mucosa of piglets around weaning from D28 to D45. CS increases expression and activity of gastric H^+-K^+-ATPase *in vivo* and *in vivo*. SS is involved in mediating the effect of CS on gastric H^+-K^+-ATPase expression and activity in weaning piglets.

Key words: Cysteamine; Weaning piglets; H^+-K^+-ATPase; Gastric mucosal cells; Somatostatin

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INTRODUCTION

The proton pump, H^+-K^+-ATPase consisting of α- and β-subunits, is the molecular base of gastric acid production and the final common pathway mediating secretion of hydrochloric acid by gastric parietal cells. The enzyme, which is typically located in the parietal cells, mediates the electroneutral exchange of intracellular H^+ and extracellular K^+ to achieve acid secretion when parietal cells are under the stimulation of secretagogues[1]. In H^+-K^+-ATPase α-

or β-subunit deficiency mice, achlorhydria and destruction of parietal cells have been observed[2,3]. The capability of gastric acid secretion is dependent on the gastric H^+-K^+-ATPase activity[4]. Therefore, H^+-K^+-ATPase activity can serve as an accurate indicator for evaluating the ability of gastric acid secretion from parietal cells.

Gastric acid secretion is regulated by stimulatory factors such as gastrin, histamine and acetylcholine, as well as inhibitory factors including somatostatin (SS). SS is a typical brain-gut-peptide releasing from D cells in the mucous membrane of stomach. Numerous publications reported that SS inhibits gastric acid secretion directly or indirectly by inhibiting the stimulatory effects of gastrin and histamine[5,6]. We found in our previous study that gastric expression of SS mRNA is upregulated in weaning piglets[7], and that gastric acid secretion is low in piglets[8]. Therefore, it is presumed that the increased inhibitory tone of SS is responsible for retarded gastric function development and insufficient gastric acid secretion which contribute, atleast partly, to diarrhea, poor growth and even death in newborn and early-weaning piglets.

Cysteamine (CS), which is able to deplete tissue SS, induces a profound loss of biological and immunological activities of SS both *in vivo* and *in vitro*[9,10]. CS is known to increase gastric acid secretion in rats, and is often used to produce the clinical model of gastric ulcer[11-13]. CS is approved to use as a feed additive in animal production to promote growth rate and improve feed efficiency[14]. However, CS application in pig production is mostly restricted to growing and fattening stages[15]. Up to now, the possible effect of CS on gastric acid secretion in weaning piglets remains unknown. Therefore, the present study was designed to examine the effect of CS on gastric acid secretion both *in vivo* and *in vitro*, the mRNA expression and activity of H^+-K^+-ATPase in gastric mucosa tissue and cultured mucosal epithelial cells were determined as response criteria. In addition, the change of SS mRNA expression in mucosal epithelial cells responding to CS exposure was also measured for elucidating the possible mechanisms underlying the CS action.

MATERIALS AND METHODS

Animals and sampling

Eighteen litters of newborn piglets from the 2nd or 3rd farrowing Xinhuai sows were employed in the *in vivo* experiment and allocated to control and treatment groups. From 12 d of age (D12), piglets in control group were fed basal diet, while the treatment group received basal diet supplemented with 120 mg/kg CS. The diet was formulated according to the requirement of piglets and provided *ad libitum*. Piglets were weaned on D35 in both groups. Six piglets from each group were slaughtered on D28 (one week before weaning), D35 (weaning), D36.5, D38, D42 and D45 (36 h, 72 h, one week and 10 d after weaning), respectively. Samples of the gastric fundic mucosa were frozen in liquid nitrogen immediately and then stored at -70 ºC until RNA extraction.

For *in vitro* experiment, four piglets at the age of D28 were killed to collect gastric mucosa for primary cell culture. DMEM (high glucose) and HEPES were products of Gibco, Hyclone, respectively. Trypsin was bought from Sigma and fetal bovine serum was purchased from Hangzhou Sijiqing Company, China.

Cells were dispersed from freshly obtained gastric mucosa of piglets as described previously[16], with minor modifications. Briefly, the gastric mucosa was washed in D-Hank's solution containing 400 U/mL penicillin, 400 μg/mL streptomycin and dipped in D-Hank's solution for 30 min. Then the tissues were dispersed by trypsin (0.15 mg/mL) at 37 °C for 1 h, filtrated and centrifuged (1 000 r/min, 5 min). Viability of the cells exceeded 95% as judged by trypan blue exclusion. Then cells at the density of 1×10^6/mL were cultured (37 °C, 50 mL/L CO_2) in a six-well plate containing DMEM (high glucose) with 10% fetal bovine serum, 15 mmol/L HEPES buffer, and 100 U/mL penicillin, 100 μg/mL streptomycin. After 24 h, the culture medium was refreshed by a new medium containing 0, 0.001, 0.01 and 0.1 mg/mL CS, respectively. The cells were continuously cultured for 20 h, and then collected for RNA extraction and H^+-K^+-ATPase activity determination.

The experiments were undertaken following the guidelines of the regional Animal Ethics Committee.

RNA extraction and analysis

Total RNA was extracted from the tissue samples with the single-step method of RNA extraction by acid guanidinium thiocyanate-phenol-chloroform[17]. Total RNA concentration was then quantified by measuring the absorbance at 260 nm in a photometer (Eppendorf Biophotometer). Ratios of absorption (260/280 nm) of all preparations were between 1.8 and 2.0. Aliquots of RNA samples were subjected to electrophoresis through a 1.4% agarose-formaldehyde gel to verify their integrity.

Two micrograms of total RNA was reverse transcribed by incubation at 42 °C for 1 h in a 25 μL mixture consisting of 10 U avian myeloblastosis virus reverse transcriptase, 10 U RNase inhibitor, 12 μmol/L random primers, 50 mmol/L Tris-HCl (pH 8.3), 10 mmol/L $MgCl_2$, 50 mmol/L KCl, 10 mmol/L DDT, 0.5 mmol/L spermidine and 0.8 mmol/L each dNTP. The reaction was terminated by heating at 95 °C for 5 min and quickly cooling on ice.

The primers for H^+-K^+-ATPase were designed according to the cDNA sequence published on GenBank (M22724): 5'-gagaaccaccacctacaag-3' as sense primer, and 5'-caacagcgaactccaag-3' as anti-sense primer, the predicted PCR product being 362 bp in size. The SS primers were designed according to the coding region of porcine SS genomic DNA sequence (GenBank, U36385): sense, 5'-agctgctgtctgaacccaac-3' and anti-sense, 5'-gaaattcttgcagccagctt-3', the expected PCR product being 161 bp in size. The PCR primers were designed using Primer Premier 5.0 and synthesized by Haojia Biotech. Ltd, China. The Quantum RNA 18S Internal Standards

kit (catalogue no. 1716, Ambion Inc., Austin, TX, USA), containing primers and competitors, was used to normalize variations in pipetting and amplification.

Different controls were set to monitor the possible contaminations of genomic DNA and environment DNA both at the stage of RT and RCR. The pooled samples made by mixing equal quantity of total RNA from all samples were used for optimizing the PCR condition and normalizing the intra-assay variations. PCR conditions were established as follows: for H^+-K^+-ATPase, the total volume of reaction was 25 μL, including 0.5 U Taq DNA polymerase (Promega, Shanghai), 5 mmol/L Tris-HCl (pH 9.0), 10 mmol/L NaCl, 0.1 mmol/L DDT, 0.01 mmol/L EDTA, 5% (w/v) glycerol, 0.1% (w/v) Triton X-100, 0.2 mmol/L each dNTP, 1.5 mmol/L $MgCl_2$, 0.7 μmol/L specific primers, 1.0–2.6 μL 18S rRNA. The program is set as: denaturation at 94 °C for 5 min, 20 cycles (for *in vivo* samples), 24 cycles (for *in vitro* samples) at 94 °C for 30 s, at 52 °C for 30 s, at 72 °C for 60 s, and a final extension at 72 °C for 8 min; for SS, the reaction mix contained 0.5 U Taq DNA polymerase, 5 mmol/L Tris-HCl (pH 9.0), 10 mmol/L NaCl, 0.1 mmol/L DDT, 0.01 mmol/L EDTA, 5% (w/v) glycerol, 0.1% (w/v) Triton X-100, 0.2 mmol/L each dNTP, 1.5 mmol/L $MgCl_2$, 1.6 μmol/L specific primers, 1.0-2.6 μL 18S rRNA. The program is set as: denaturation at 94 °C for 5 min, 26 cycles at 94 °C for 30 s, at 54 °C for 30 s, at 72 °C for 30 s, and a final extension at 72 °C for 8 min. All samples were included in the same run of PCR on GeneAmp PCR system 9600 (Perkin Elmer, USA) and repeated atleast thrice.

Twenty microliters of PCR products was analyzed by 2% agarose gel electrophoresis. The gels were stained with ethidium bromide and photographed with a digital camera. The net intensities of individual bands were measured using Kodak Digital Science 1D software (Eastman Kodak Company, Rochester, NY, USA). The ratio of band density for target genes to that for 18S rRNA was used to represent the abundance of H^+-K^+-ATPase and SS mRNA expression.

H^+-K^+-ATPase activity assay

H^+-K^+-ATPase activity was measured according to the method described by Hervatin *et al*.[18]. H^+-K^+-ATPase activity was evaluated as the amount of inorganic phosphate released from ATP by the method of Sanui[19]. The reaction was initiated at 37 °C by addition of 2 mmol/L ATP-Mg^{2+} salt as substrate and proceeded in a total volume of 1.0 mL containing 60 mmol/L Tris-1,4-piperzine-bis (ethanesulfonic acid) (pH 7.4), 0.1 mmol/L ouabain, 90 mmol/L sucrose, 100 μL sample and either 15 mmol/L KCl or 30 mmol/L sucrose. It was terminated after 10 min by addition of 1.5 mL of ice-cold 14% trichloroacetic acid. Ouabain was included to avoid Na^+-K^+-ATPase activity. The ATPase activity measured without K^+ was taken as the basal Mg^{2+}-ATPase activity. The difference between the activities measured with and without K^+ was defined as H^+-K^+-ATPase activity. Bradford[20] assay was employed to determine the tissue protein content and the activity of H^+-K^+-ATPase was expressed as the amount of inorganic phosphate released per milligram of protein per hour (μmol Pi/mg prot/h).

Statistical analysis

All data were expressed as mean±SE. The data were analyzed by *t*-test for independent samples or ANOVA with Statistical Packages for the Social Sciences (2000). $P<0.05$ was considered statistically significant.

RESULTS

Expression and activity of H^+-K^+-ATPase in vivo

H^+-K^+-ATPase mRNA expression and H^+-K^+-ATPase activity in control group exhibited a trend to increase from D28 to D45, reaching a peak on D45, but did not show significant age differences (Figure 1). Furthermore, neither the mRNA expression nor the activity of H^+-K^+-ATPase was affected significantly by weaning.

Effects of CS on expression and activity of H^+-K^+-ATPase in vivo

As shown in Figure 1, CS increased the level of H^+-K^+-

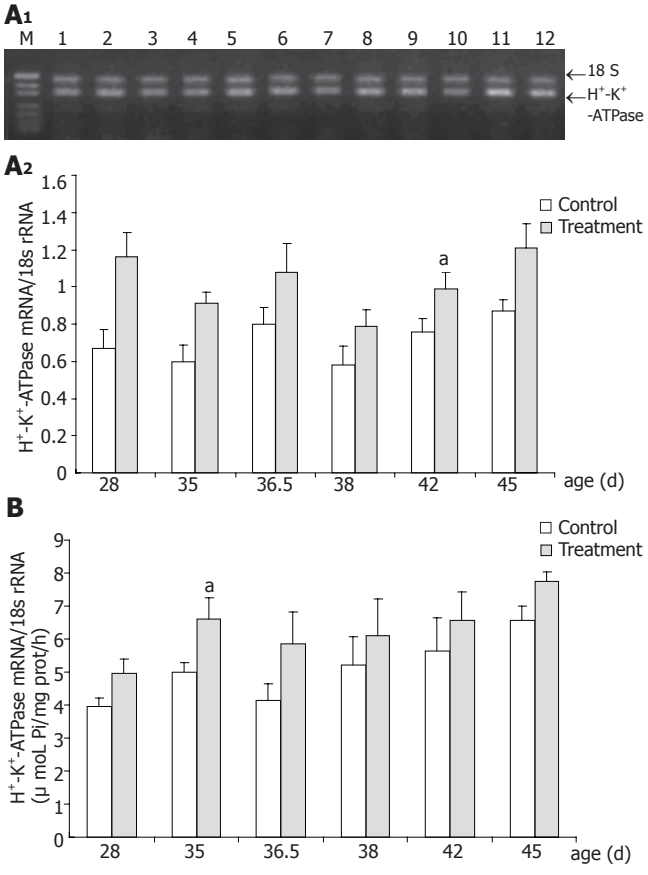

Figure 1 Expression (**A**1-2) and activity (**B**) of H+-K+-ATPase in vivo. Lane M: PUC19 marker; lanes 1-6: piglets at 28, 35, 36.5, 38, 42, 45 d of age in control group, respectively; lanes 7-12: piglets at 28, 35, 36.5, 38, 42, 45 d of age in treatment, respectively. Bars without a common superscript representing significant differences between age groups (small letters a and b for control group and capital letters A and B for treatment group); $^aP<0.05$ indicating differences between control and treatment groups at the same age.

ATPase mRNA expression and the differences were significant on D28 (P = 0.014), D35 (P = 0.017), D42 (P = 0.013) and D45 (P = 0.046), respectively. Relative abundances of H^+-K^+-ATPase mRNA expression were significantly increased by 73%, 53%, 30%, and 39% in treatment group compared with control at the same age. CS supplementation increased markedly H^+-K^+-ATPase activity by 32.3% on D35 (P = 0.043) and 18.3% on D45 (P = 0.040), respectively, compared with that of the control counterparts.

Effects of CS on expression and activity of H^+-K^+-ATPase in vitro

As shown in Figure 2, low concentration of CS exhibited no effects on both mRNA expression and activity of H^+-K^+-ATPase, while moderate and high concentrations of CS markedly increased H^+-K^+-ATPase mRNA expression by 61% (P = 0.03) and 65% (P = 0.014), and H^+-K^+-ATPase activity by 48% (P = 0.017) and 50% (P = 0.022), respectively.

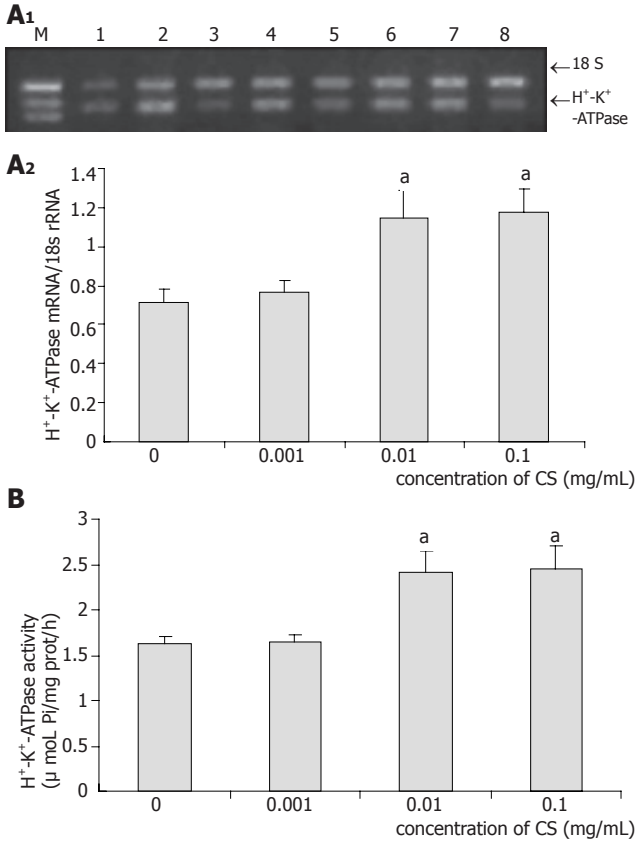

Figure 2 Effect of CS on expression (**A**$_{1\text{-}2}$) and activity (**B**) of H+-K+-ATPase in vitro. Lane M: PUC19 marker, lanes 1-2: control; lanes 3-4: low concentration group; lanes 5-6: moderate concentration group; lanes 7-8: high concentration group. [a]P<0.05 *vs* 0 mg/mL of CS.

Effects of CS on SS mRNA expression in vitro

Low concentration of CS had no influence on SS mRNA expression, whereas SS mRNA expression was markedly increased by 56% (P = 0.024) in moderate concentrations of CS and 64% (P = 0.022) in high concentrations of CS (Figure 3).

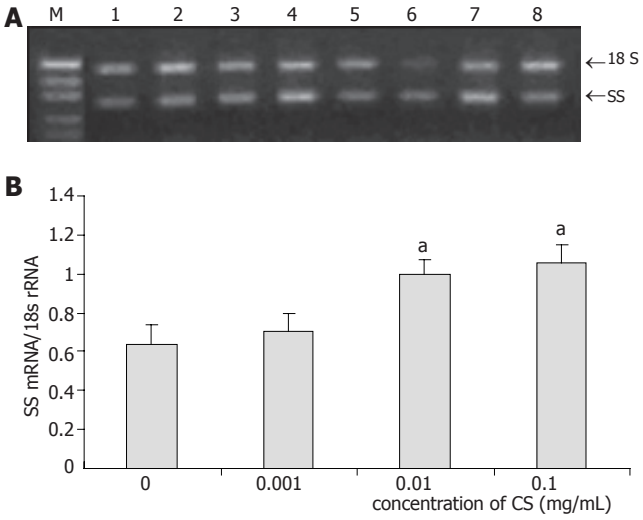

Figure 3 Effect of CS on SS mRNA expression *in vitro*. Lane M: PUC19 marker, lanes 1 and 2: control; lanes 3 and 4: low concentration group; lanes 5 and 6: moderate concentration group; lanes 7 and 8: high concentration group (**A**,**B**). [a]P<0.05 *vs* 0 mg/mL of CS.

DISCUSSION

The developmental pattern of H^+-K^+-ATPase was found to be in agreement with that of gastric acid secretion capacity, both at the level of mRNA expression and enzyme activity. Yang *et al*.[21] reported that H^+-K^+-ATPase mRNA expression and activity in fundic gland keep increasing with age from gestational day 19.5 to week 18 of postnatal age in rats. The developmental pattern of human gastric H^+-K^+-ATPase from week 25 of gestation by Western blot analysis agrees with that of gastric pH recorded in preterm infants[22]. Furthermore, the H^+-K^+-ATPase mRNA content in rat fundus increases with age from one week to six weeks of age and the change parallels the developmental change of acid secretion capacity[23]. However, the developmental pattern of H^+-K^+-ATPase expression and activity in pigs has not been reported. In contrary to the reported data, the present study failed to show significant developmental change in both the expression and the activity of H^+-K^+-ATPase in piglets around weaning throughout the period of observation. It seems that the developmental pattern of H^+-K^+-ATPase is species-specific. In addition, it is documented that gastrin is involved in the regulation of H^+-K^+-ATPase expression. In ovine fetus, developmental pattern of gastrin mRNA agrees with that of H^+-K^+-ATPase expression[24]. Gastrin can stimulate H^+-K^+-ATPase expression[25] and improve its activity by increasing the intracellular Ca^{2+} concentration to initiate the phosphorization of H^+-K^+-ATPase in parietal cells[26]. In accordance with our findings, gastrin contents in gastric tissues are stable from 4 to 6 wk of age in piglets[27].

Since the period of observation was limited to less than 3 wk from D28 to D45 in the present study, the possibility that H^+-K^+-ATPase expression and activity subjected to change during growth in a longer term cannot be excluded.

Until now no report is available describing the effect of weaning stress on expression and activity of H^+-K^+-ATPase. Our results indicated that weaning stress exhibited no effect on expression and activity of H^+-K^+-ATPase, which might be attributed to the relatively mature age of weaning on D35. This explanation is supported by the study of Efird *et al*.[28], who found that gastric acid secretion of piglets weaned after 35 d of age is not easily affected by weaning.

Our earlier publication reported that the inhibition of gastric acid secretion agrees with the upregulation of gastric SS expression in pre-weaning piglets[7]. Read *et al*.[24] reported that the developmental pattern of gastric SS mRNA is on the contrary to the patterns of gastrin mRNA and H^+-K^+-ATPase mRNA in ovine. *In vitro* studies have provided further evidence that endogenous SS plays a role as a strong inhibitory factor in gastric acid secretion, since SS antiserum significantly increases gastric acid release from perfused stomach of sheep[29].

CS possesses the ability to deplete tissue SS. Szabo and Reichlin[9] found that, in rats, CS administration brings about a prompt depletion of radioimmunoassayable SS in plasma, stomach, duodenum, pancreas, and hypothalamus. Some researches showed that gastrin plays a significant role in CS-induced hypersecretion of gastric acid. Intravenous infusion of CS in the perfused rat stomach results in a significant increase in acid secretion, which is accompanied with a marked increase in plasma gastrin concentration. The injection of anti-gastrin rabbit serum completely blocks CS-induced acid increase, and infusion of a gastrin receptor antagonist also suppresses CS-induced increase in acid secretion[13]. Van de Brug *et al*.[12] also found that intravenous bolus administration of CS induces increase in serum gastrin concentration and gastric acid outputs. However, there is no report concerning the effect of CS on expression and activity of H^+-K^+-ATPase. In the present study, we found that CS significantly increased both the expression and activity of H^+-K^+-ATPase *in vivo*. To further affirm the effect of CS on H^+-K^+-ATPase, we added CS to the culture medium of gastric mucosal epithelial cells *in vitro*. The results indicated that moderate and high concentrations of CS increased significantly both the expression and activity of H^+-K^+-ATPase, accompanied with a marked increase in the expression of SS. The upregulation of SS mRNA expression might be the consequence of SS depletion. The signal of SS depletion feeds back to the cells to boost SS synthesis in order to maintain homeostasis. Kanayama and Liddle[30] also found that the content of SS mRNA in duodenum is reduced after SS perfusion in rats. However, this upregulation might be temporal, since SS mRNA in stomach and brain significantly increases, then reduces after perfusion of CS in rats[31]. These authors presumed that SS mRNA upregulation results from SS depletion, and the subsequent SS mRNA downregulation is caused by direct effect of CS on SS expression.

In conclusion, the present experiments provide evidence that the mRNA expression and activity of H^+-K^+-ATPase in gastric mucosa remain relatively constant in piglets around weaning from D28 to D45. CS increases gastric expression and activity of H^+-K^+-ATPase both *in vivo* and *in vitro*. In addition, SS is involved in mediating the effect of CS on gastric H^+-K^+-ATPase expression and activity in weaning piglets, although the complex effect of CS on SS mRNA expression awaits further investigation.

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• *BRIEF REPORTS* •

Hepatitis E virus chimeric DNA vaccine elicits immunologic response in mice

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Abstract

AIM: To construct the plasmid pcHEV23 containing fragments of HEV ORF2 and ORF3 chimeric gene and to assess its ability to elicit specific immunologic response in mice.

METHODS: The gene encoding the structural protein of HEV ORF2 fragment and full-length ORF3 was amplified by PCR. The PCR products were cloned into an eucaryotic expression plasmid pcDNA3. The resulting plasmid pcHEV23 was used as a DNA vaccine to inoculate BALB/c mice intramuscularly thrice at a dose of 100 or 200 μg. Mice injected with empty pcDNA3 DNA or saline served as control and then specific immune responses in the mice were detected.

RESULTS: After 2-3 times of inoculation, all mice injected with pcHEV23 had anti-HEV IgG seroconversion and specific T lymphocyte proliferation. The lymphocyte stimulation index in the group immunized with pcHEV23 (3.1±0.49) was higher than that in the control group (0.787±0.12, $P<0.01$). None in the control group had a detectable level of anti-HEV IgG.

CONCLUSION: DNA vaccine containing HEV ORF2 and ORF3 chimeric gene can successfully induce specific humoral and cellular immune response in mice.

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INTRODUCTION

Hepatitis E virus (HEV) is an unclassified, non-enveloped RNA virus, a causative agent of acute hepatitis E transmitted principally via the fecal-oral route. The virus can cause large water-borne epidemics of the disease and sporadic cases as well. Hepatitis E occurs predominantly in developing countries usually affecting young adults with a fatality rate of 15-20% in pregnant women[1]. However, no effective treatment is currently available for hepatitis E and there are no commercial vaccines for hepatitis E in the world. Although at least four major genotypes of HEV have been identified, only one serotype of HEV is recognized. DNA vaccine can synthesize viral proteins within the host cells and induce humoral and cellular immune responses[2,3]. In this study, we constructed eucaryotic expression plasmid containing HEV ORF2 fragment and full-length ORF3 (DNA vaccine) chimeric gene and inoculated it to BALB/c mice to detect the specific humoral and cellular immune responses in mice.

MATERIALS AND METHODS

Construction of plasmid

All PCR primers were designed according to the nucleotide sequence of a Chinese HEV isolate (DDBJ accession number D11092)[4]. HEV mRNA was extracted from the feces of a patient with hepatitis E in Hangzhou, Zhejiang Province. The HEV ORF2 fragment and full-length ORF3 chimeric gene were amplified by RT-PCR. The PCR product was inserted into an eucaryotic expression plasmid pcDNA3 to form a recombinant plasmid pcHEV23 (DNA vaccine) (Figure 1).

DNA inoculation protocol

Thirty-two female BALB/c mice (18-20 g) provided by Zhejiang Experimental Animal Center were used for immunization and divided into four groups: Group 1 was injected with 100 μL saline solution as control. Group 2 was injected with 100 μg/100 μL vector pcDNA3 as control. Group 3 was injected with 100 μg/100 μL pcHEV23 plasmid. Group 4 was injected with 200 μg/100 μL pcHEV23 plasmid. Three weeks after the first injection, mice were bled and then boosted by same method. After another 3 wk, mice were boosted again for the second time (Table 1).

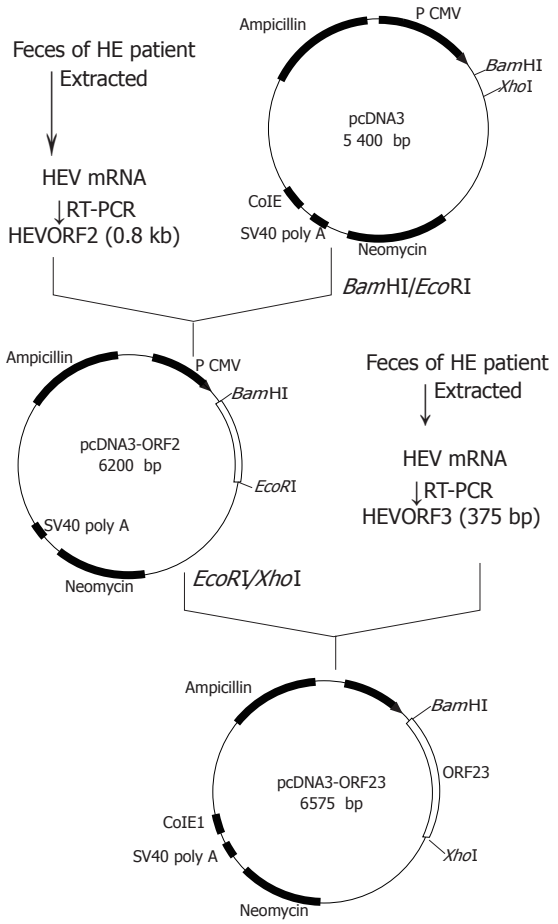

Figure 1 Construction of pcHEV23 plasmid (pcDNA3-ORF23).

Table 1 Protocol of DNA injection

Group	Mice (*n*)	Immunogen	Time of injection (wk)		
1	8	Saline	0	3	6
2	8	pcDNA3(100 μg/100 μL)	0	3	6
3	8	pcHEV23(100 μg/100 μL)	0	3	6
4	8	pcHEV23(200 μg/100 μL)	0	3	6

Serological test

Two weeks after the first three injections, blood samples were collected. All serum specimens were tested for anti-HEV IgG by EIA. In brief, microwell plates (Nunc, Roskilde, Denmark) were coated overnight at 4 °C with purified HEV ORF23 proteins (expressed *in E coli*) at 1 μg/mL in carbonate–bicarbonate buffer (pH 9.6). The wells were washed thrice with 0.05% Tween 20 in PBS (PBS-T) and then blocked with 2% BSA in PBS-T at 37 °C for 1 h. Following three washes with PBS-T, serum samples diluted in 2% BSA were added to the plates and incubated at 37 °C for 1 h. Following five washes, HRP-conjugated anti-human IgG (Sigma) diluted 2 000-fold in PBS-T was added to detect the bound antibodies. Following incubation at 37 °C for 1 h, the plates were washed as above and the substrate tetramethylbenzidine solution (Sigma) was added to the wells. After incubation at room temperature for 15 min, color development was stopped by adding 2 mol/L H_2SO_4. Optical density (OD) at 450 nm was determined with an ELISA reader. The cut-off values were set for each test as 2.1 times the mean value of negative control samples.

Table 2 Anti-HEV IgG titers after the third injection (mean±SD)

Group	1	2	3	4
Mean±SD	0.116±0.009[b]	0.210±0.028[b]	0.353±0.085[b]	0.336±0.066[b]

[b]$P<0.01$ *vs* control.

Table 3 Result of assay of T-cell proliferation (mean±SD)

Group	1	2	3	4
	0.787±0.12[b]	1.54±0.25[b]	3.1±0.49[b]	2.85±0.59[b]

[b]$P<0.01$ *vs* control.

T-lymphocyte proliferation

All the animals were killed 14 d after the last injection to analyze cellular immune responses. Single cell suspension of splenocytes was prepared for each individual animal. Splenocytes were immediately cultured in the presence of HEV ORF23 (20 μg/mL, expressed in *E coli*). Sixty-eighth day after culture, MTT was then added to the cells to measure antigen-specific proliferation. Lymphocyte stimulation index (SI) was measured according to formula: SI = A_{570} (antigen stimulation)/A_{570} (control).

RESULTS

Construction of pcHEV23 plasmid

The sequencing data indicated that the pcHEV23 construct (Figure 1) contained the correct orientation of the HEV ORF2 and ORF3 fragments (sequencing data not shown).

Analysis of anti-HEV response in pcHEV23-inoculated mice

None of the mice had seroconversion after the initial injection of pcHEV23. Following the third dose of pcHEV23, anti-HEV IgG titers in groups 3 and 4 was higher than those in groups 1 and 2 ($P<0.01$, Table 2).

T-lymphocyte proliferation assay

The lymphocyte SI in the group immunized with pcHEV23 was higher than that in saline group and pcDNA3 in control DNA group ($P<0.01$). Results are shown in Table 3.

DISCUSSION

DNA vaccines represent a new and potentially powerful approach for the development of subunit vaccines. DNA vaccines induce a broad range of immune responses due to efficient priming of T lymphocytes[5]. This novel approach to vaccination is attractive as it offers several

desirable features. First, DNA is not infectious and does not replicate and encodes only the protein or proteins of interest. Second, DNA is stable and can be made inexpensively in large quantities at a high level of purity. Third, plasmid DNA does not contain any heterologous protein components compared to a recombinant virus vaccine. Fourth, DNA vaccine can induce both cell-mediated and humoral immunity. Finally, antigen expression persists after DNA vaccination, promoting the induction of long-lived memory immune cells[6]. DNA immunization can be defined as a physical delivery of nucleic acids *in vivo* to express antigenic proteins and elicit specific immune responses. Direct naked DNA inoculation obviates the requirement of purified antigens. Pathogenic antigens synthesized inside the inoculated host cells can be processed in a natural form to develop classes I and II-regulated immune responses, mimicking the aspects of live attenuated virus.

Epitope mapping demonstrates that there are at least 7 immunodominant epitopes in HEV ORF2 region and four epitopes in HEV ORF3 region[7,8]. In this study, the full length of HEV ORF3 cDNA and partial HEV ORF2 cDNA were combined and used as an antigen-coding sequence for construction of HEV DNA vaccine.

In conclusion, direct injection of pcHEV23 is able to induce specific anti-HEV IgG and T-lymphocyte proliferation. HEV DNA vaccine constructed by us can successfully induce both humoral and cellular immune responses and appears to be a viable alternative to the recombinant protein subunit vaccine candidate.

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• *BRIEF REPORTS* •

Relation of overexpression of S phase kinase-associated protein 2 with reduced expression of p27 and PTEN in human gastric carcinoma

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Abstract

AIM: To investigate the significance of S phase kinase-associated protein 2 (Skp2) expression in human gastric carcinoma and the relation between expressions of Skp2, p27 and PTEN.

METHODS: Immunohistochemical analysis was performed on 138 gastric carcinoma specimens, their paired adjacent mucosa specimens, 102 paired lymphatic metastatic carcinoma tissue specimens, 30 dysplasia specimens, 30 intestinal metaplasia specimens, 10 chronic superficial gastritis specimens and 5 normal gastric mucosa specimens for Skp2 expression and on 138 gastric carcinoma specimens for p27 and PTEN expression.

RESULTS: Skp2 labeling frequency was significantly higher in intestinal metaplasia (12.68±0.86) and adjacent mucosa (19.32±1.22) than in normal gastric mucosa (0.53±0.13) and chronic superficial gastritis (0.47±0.19) (P = 0.000); in dysplasia (16.74±0.82) than in intestinal metaplasia (P = 0.000); in gastric primary carcinoma (31.34±2.17) than in dysplasia and adjacent mucosa (P = 0.000); in metastasis gastric carcinoma in lymph nodes (39.76±2.00) than in primary gastric carcinoma (P = 0.037), respectively. Skp2 labeling frequency was positively associated with differentiation degree (*rho* = 0.315, P = 0.000), vessel invasion (*rho* = 0.303, P = 0.000) and lymph node metastasis (*rho* = 0.254, P = 0.000) of gastric cancer. Expression of Skp2 was negatively associated with p27 (*rho* = -0.451, P = 0.000) and PTEN (*rho* = -0.480, P = 0.000) expression in gastric carcinoma. p27 expression was positively associated with PTEN expression in gastric carcinoma (*rho* = 0.642, P = 0.000).

CONCLUSION: Skp2 overexpression may be involved in carcinogenesis and progression of human gastric carcinoma *in vivo*, possibly via p27 proteolysis. PTEN may regulate the expression of p27 by negatively regulating Skp2 expression.

Key words: Gastric carcinoma; Skp2; p27; PTEN

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INTRODUCTION

Dysregulation of the cell cycle is required for the formation of most malignant tumors. Progression of the cell cycle is controlled by interactions between cell cycle control proteins (cyclins) and their catalytically active cyclin-dependent kinase (CDKs). The activity of each cyclin-CDK complex is in turn regulated by several different mechanisms; the most important being negative regulation by CDK inhibitors[1]. p27 is an inhibitor of cyclinE-CDK2 and cyclinA-CDK2, which drive cells from G_1 to S phase of the cell division cycle[2,3]. Loss of p27 function therefore accelerates cell cycle progression and predisposes cells to malignant transformation, as is well illustrated by the observation of increased tumor incidence in hemizygous and homozygous p27-deleted mutant mice after carcinogen exposure[4,5]. Many clinical studies also indicate that low levels of p27 are associated with high aggressiveness and poor prognosis in a large variety of malignant tumors[2,3], including breast carcinoma[6,7], colorectal carcinoma[8], lung cancer[9], prostate cancer[10] and gastric carcinoma[11].

The amount of p27 is mainly regulated by post-translational ubiquitin-proteasome-mediated proteolysis[12]. Cell cycle-dependent degradation of p27 is dependent on phosphorylation at Thr^{187} in late G_1 phase by CDK2 under positive regulation by cyclinE. Thr^{187} phosphorylation is a necessary prerequisite for the sequential addition of ubiquitin molecules by an ubiquitin ligase complex, SCF^{skp2} composed of Skp1, Cull, Rbx1 and the F-box protein Skp2[3]. Polyubiquitination of p27 then targets p27 for degradation in proteasome, thus removing the p27 cell

cycle "brake" and allowing cells to transition from G_1 to S phase[13].

Some investigations have shown that Skp2 is a specific substrate-recognition subunit of SCF^{skp2}, expression of Skp2 is required for the ubiquitination and subsequent degradation of p27 *in vitro*[20-22], and Skp2 knock-out cells show high levels of p27[16]. The level of p27 has also been reported to be inversely related to that of Skp2 in lymphoma[24], oral squamous cell carcinoma[18,19] and colorectal carcinoma[20].

Recently, Mamillapalli *et al*.[21] reported that PTEN, a tumor suppressor, regulates the ubiquitin-dependent degradation of p27 through SCF^{Skp2}. Yang *et al*.[22] suggested that induction of Skp2 may be causally linked with decreased levels of p27 in prostate cancer and implicate PTEN in the regulation of Skp2 expression *in vivo*.

Recent studies have shown that Skp2 has oncogenic potential in breast epithelial cells and is overexpressed in a subset of breast carcinomas (ER- and Her-2 negative)[23] and Skp2 can mediate transformation and is upregulated during oral epithelial carcinogenesis[18]. Recently, a line of evidence also indicates a possible relationship between Skp2 expression and the malignancy of tumors. Skp2 expression has been shown to be greatly increased in malignantly transformed cell lines including oral squamous cell carcinoma[18] and correlates directly with the grade of malignancy of lymphoma[17] and oral squamous cell carcinoma[18]. Kudo *et al*.[19] reported that high Skp2 expression is also correlated with poor prognosis in oral squamous cell carcinoma. Thus, Skp2 may have a great significance in human carcinogenesis. However, few studies are available regarding the significance of Skp2 expression in human gastric carcinomas *in vivo* and there are no studies regarding the relationship between Skp2, p27 and PTEN expression. We therefore investigated the significance of Skp2 expression in human gastric carcinomas and the relationship between Skp2, p27 and PTEN expression *in vivo*.

MATERIALS AND METHODS

Patients and gastric specimens

One hundred and thirty-eight surgically resected gastric carcinoma specimens, paired adjacent mucosa specimens and paired 102 lymphatic gastric carcinoma tissue specimens metastatically selected from the Fourth Affiliated Hospital of Hebei Medical University were used in this study. All gastric carcinoma patients underwent total or subtotal gastrectomy and no patient received any treatment for cancer before surgery. All regional lymph nodes were removed. The patients comprised 111 males and 27 females with a mean age of 58.5 years and a median age of 60 years (from 36 to 78 years). After surgery, gastric specimens were fixed in 40 g/L neutral-buffered formaldehyde and embedded in paraffin. Additionally, 75 biopsy cases (5 cases of normal gastric mucosa, 10 cases of chronic superficial gastritis, 30 cases of intestinal metaplasia and 30 cases of dysplasia) were included in this study as well. To avoid evaluator variability, all pathological diagnoses were done by two pathologists. Clinical stage was done according to the International Union Contrele Cancer criteria published in 1997.

Immunohistochemistry

A standard Non-Biotin HRP two-step immunohistochemical method (Zymed) was used. Four-micrometer-thick sections were deparaffinized and rehydrated. Endogenous peroxidase in sections was inactivated in 30 mL/L hydrogen peroxide (H_2O_2) in ethanol for 15 min at room temperature. The sections were washed thrice for 5 min with 0.01 mol/L phosphate-buffered saline (PBS) and then heated in a citrate buffer (0.01 mol/L, pH 6.4) for PTEN and p27 or in a EDTA buffer (1 mmol/L, pH 8.0) for Skp2 in an 800-W microwave oven for 12 min for antigen retrieval. The sections were incubated with mouse monoclonal antibody to Skp2 (1:50 dilution, Zymed), p27 (1:50 dilution, Santa Cruz) and PTEN (1:50 dilution, Zymed) overnight at 4 °C, washed thrice for 5 min with 0.01 mol/L PBS 3 and then incubated with common IgG (Fab fraction)-HRP complex for 30 min at room temperature. Immunoreactive products were visualized using 3,3'-diaminobenzidine/H_2O_2, and finally the sections were counterstained with hematoxylin. Some sections were incubated with PBS instead of primary antibody as negative control to verify the specificity of the immunoreactions. Vascular endothelial cells showed strong PTEN expression with a nuclear predominance and served as an internal positive control for PTEN in this study. The positive breast adenocarcinoma served as a positive control for Skp2 and p27.

Immunohistochemical quantitation

Immunostaining of nuclear Skp2, p27 and PTEN in each specimen was evaluated microscopically and recorded as the percentage of Skp2, p27, and PTEN-positive cells (labeling frequency), after at least 1 000 nuclei at the lesion site were calculated in at least five high-power fields (×400). All specimens were evaluated without any knowledge of the patients' clinical information.

Statistical analysis

The differences in Skp2 labeling frequencies among specimens of normal gastric mucosa, chronic superficial gastritis, intestinal metaplasia, dysplasia, gastric adenocarcinoma, adjacent mucosa and lymphatic metastatic gastric carcinoma were compared by Mann-Whitney *U* test. The relation between Skp2 expression and the clinical and pathological variables was evaluated using the Spearman correlation coefficient. The Spearman's correlation coefficient testing was also used to determine the relation between Skp2, PTEN and p27 as well as between PTEN and p27. $P<0.05$ was considered statistically significant. All analyses were performed using SPSS 11.0 statistical software.

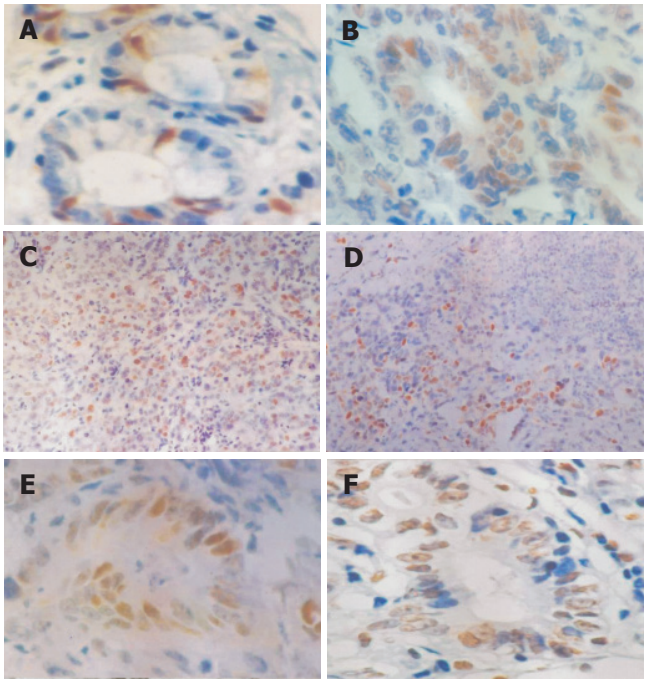

Figure 1 Skp2, p27 and PTEN expression in different gastric tissues (DAB and hematoxylin stain). **A:** Positive Skp2 in nuclei of intestinal metaplasia and dysplasia cells (original magnification ×400); **B:** Positive Skp2 in nuclei of well-differentiated gastric carcinoma cells (original magnification ×400); **C:** Positive Skp2 in nuclei of poorly differentiated gastric carcinoma cells (original magnification ×100); **D:** Positive Skp2 in lymphatic metastatic gastric carcinoma cells (original magnification ×100); **E:** Positive p27 in nuclei of well-differentiated gastric carcinoma cells (original magnification ×400); **F:** Positive PTEN in nuclei of well-differentiated gastric carcinoma cells (original magnification ×400).

RESULTS

Expression of Skp2 in human gastric specimen

The Skp2 immunoreactivity was predominantly localized in the nuclei of gastric cells (Figure 1). In 5% of the cancer specimens examined, a weak or moderate cytoplasmic immunoreactivity could be seen in addition to the predominant nuclear reactivity in cancer cells. Skp2 labeling frequency in normal gastric mucosa, intestinal metaplasia, dysplasia, primary gastric carcinoma and lymphatic metastatic gastric carcinoma was significantly higher than in normal gastric mucosa and chronic superficial gastritis ($P = 0.000$); it was in dysplasia than in intestinal metaplasia ($P = 0.000$); in primary gastric carcinoma than in dysplasia ($P = 0.000$); in lymphatic metastatic gastric carcinoma than in primary gastric carcinoma ($P = 0.037$, Table 1).

Relation between Skp2 labeling frequency and clinicopathological features in gastric carcinoma

Relation between Skp2 labeling frequency and clinicopathological variables including age, gender, histological differentiation, depth of invasion, vessel invasion, lymphatic metastasis, distant metastasis as well as clinical stage is summarized in Table 2. Skp2 labeling frequency was negatively associated with age, gender, depth of invasion, distant metastasis as well as clinical stage. A significant correlation was found between the

Table 1 Skp2 protein expression in human gastric tissues

Tissue	Specimens (*n*)	Skp2 labeling frequency(%) Median	Mean±SE
Normal gastric mucosa	5	0.00	0.47±0.19
Chronic superficial gastritis	10	0.00	0.53±0.13
Intestinal metaplasia	30	12.00	12.68±0.86
Dysplasia	30	19.00	16.74±0.82
Primary gastric carcinoma	138	29.00	31.34±2.17
Adjacent mucosa	138	16.00[6]	19.32±1.22
Metastasis tumor tissue in lymph node	102	39.50[7]	39.76±2.00

[1,2,3,4]Frequency was significantly higher than in normal gastric tissue and chronic superficial gastritis ($P = 0.000$,all the same; Mann-Whitney U test). [2]Frequency was significantly higher than in intestinal metaplasia ($P = 0.000$; Mann-Whitney U test). [3]Frequency was significantly higher than in intestinal metaplasia and dysplasia, ($P = 0.000$,all the same; Mann-Whitney U test). [5]Frequency was Significantly higher than in primary tumors (P = 0.037; Mann-Whitney U test).

Table 2 Skp2 protein expression in 138 human gastric cancers

Clinicopathological feature	Specimens (*n*)	Skp2 labeling frequency (%) Mean±SE	rho	*P*
Patients	138	36.32±1.22		
Age (yr)	138	58.50	0.190	0.833
<60	66	34.25±3.58		
⩾60	72	34.41±2.63		
Gender			0.119	0.179
Females	27	24.96±5.21		
Males	111	32.90±2.37		
Differentiation grade			0.315	0.000
Well/Moderate	63	22.41±2.59		
Poor	75	38.85±3.10		
Depth of invasion			0.001	0.986
Serosal	24	25.84±2.45		
Outside soft tissue	114	29.25±4.87		
Vessel invasion			0.303	0.000
Absent	120	27.89±2.11		
Present	18	54.39±6.84		
Lymph node metastasis			0.254	0.000
Absent	36	27.94±3.38		
Present	102	37.14±2.69		
Distant metastasis			0.091	0.307
Absent	26	22.77±6.92		
Present	12	31.79±2.38		
Clinical stage			0.069	0.440
0	0			
Ia	0			
Ib	3	22.78±6.92		
II	36	29.81±3.14		
IIIa	12	44.67±6.78		
IIIb	66	34.50±3.55		
IV	9	53.00±0.57		

[1,2,3]Skp2 labeling frequency was positively correlated with differentiated degree (rho = 0.315, $P = 0.000$), vessel invasion (rho = 0.303, $P = 0.000$), and lymph node metastasis (rho = 0.254, $P = 0.000$) in gastric carcinoma, respectively. [4]Because clinical staging could not be performed due to the lack of accurate records about lymph node in operation, data of 12 cases were cancelled in statistical analysis.

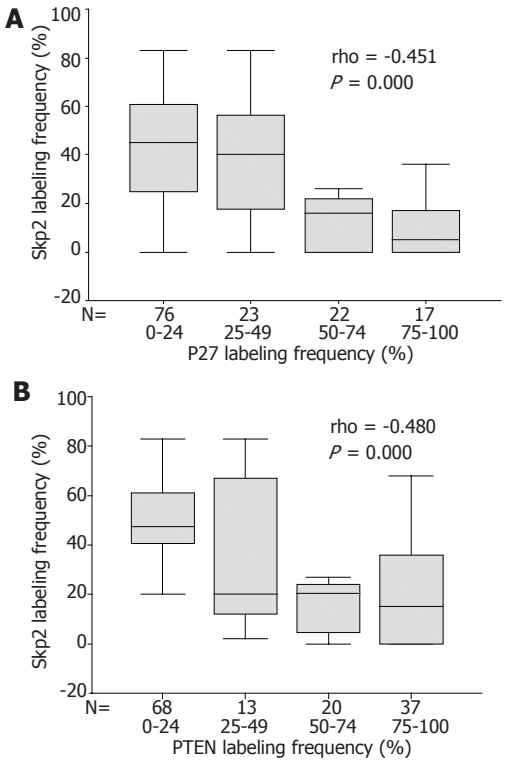

Figure 2 Correlation of Skp2 with p27 (**A**) and PTEN (**B**) protein levels. Each box and the associated bars represent the values of middle 50% and the range of the data respectively. The dark line within a box denotes the median.

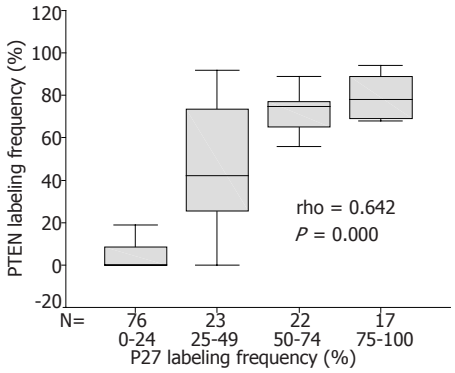

Figure 3 Correlation of p27 and PTEN expression in human gastric carcinoma. Each box and the associated bars represent the values of middle 50% and the range of the data respectively. The dark line within a box denotes the median.

Skp2 labeling frequency and differentiation degree (*rho* = 0.315, *P* = 0.000), vessel invasion (*rho* = 0.303, *P* = 0.000) and lymphatic metastasis (*rho* = 0.254, *P* = 0.000). Poorly differentiated gastric carcinoma, gastric carcinoma with vessel invasion and gastric carcinoma with lymphatic metastasis tended to have a higher Skp2 labeling frequency.

Relation between Skp2, p27 and PTEN expression in gastric carcinoma

Given the biochemical link between Skp2 and p27, we asked whether there was a correlation between Skp2 and p27 levels in gastric carcinoma using the same set of gastric carcinoma specimens used for the Skp2 analysis. A statistically significant inverse relation between p27 and Skp2 labeling frequency was evident (*rho* = -0.451, *P* = 0.000; Figure 2A). *In vitro* study linking loss of PTEN with increased Skp2 levels led us[29] to also compare the expression of PTEN and Skp2 using the same set of gastric carcinoma specimens. Interestingly, we found that increased Skp2 expression was significantly correlated with loss and decrease of PTEN expression in gastric carcinoma (*rho* = -0.480, *P* = 0.000; Figure 2B). Additionally, a positive relation between p27 and PTEN expression was observed in gastric carcinoma (*rho* = 0.642, *P* = 0.000; Figure 3).

DISCUSSION

Protein degradation by the ubiquitin–proteasome pathway plays a fundamental role in the regulation of eukaryotic cell cycle. Proteolysis of G_1 regulatory proteins is mediated by SCF ubiquitin ligase complex (SCF^{Skps}) composed of four major subunits, Skp1, Cul1, Rbx1/Roc1 and one of the F-box proteins (Fbps)[24]. There are 11 Fbps in budding yeast, 22 in Drosophila[25] and 38 in human beings[26,27]. Skp2 is a Fbp first identified together with Skp1 as an interactor of the cyclinA-Cdk2 complex which drives cells from G_1 to S phase, hence it is named as S phase kinase-associated protein 2 (Skp2)[24].

In this study, we found that Skp2 immunoreactivity was rare in normal gastric mucosa and chronic superficial gastritis, but Skp2 labeling frequency was significantly increased during the course of intestinal metaplasia, dysplasia and primary gastric carcinoma. From pathological point of view, development of gastric adenocarcinoma involves progression through a well-defined series of histological steps initiated by the change of normal mucosa to chronic superficial gastritis, followed by the appearance of atrophic gastritis and intestinal metaplasia, then dysplasia and finally adenocarcinoma[28]. These results suggest that Skp2 may have oncogenic potential in gastric carcinoma and is involved in gastric carcinogenesis. It may become a new biomarker in gastric carcinogenesis. Additionally, we found that Skp2 labeling frequency in adjacent mucosa of gastric carcinoma was significantly higher than that in normal gastric mucosa, suggesting that some adjacent mucosae of gastric carcinoma have normal histological structure, but the expression of some proteins is changed.

In this study, we found that Skp2 labeling frequency was positively correlated with differentiation degree, lymphatic metastasis and vessel invasion of gastric carcinoma. Poorly differentiated gastric carcinoma and gastric carcinoma with lymphatic metastasis and vessel invasion tended to have a higher Skp2 labeling frequency. Skp2 labeling frequency was higher in lymphatic metastatic gastric carcinoma than in primary gastric carcinoma. The overexpression of Skp2 could modulate the malignant phenotype of gastric carcinoma cells, suggesting that overexpression of Skp2 may be associated with metastasis

potential of gastric carcinoma cells. Further studies need to be done to clarify their mechanism.

In the present study, we also found that Skp2 expression was inversely correlated with the expression of p27, which is consistent with the results reported in other studies[18,19,21]. These results suggest that Skp2 overexpression is associated with p27 protein degradation in cancer tissue, which may be a reason why Skp2 overexpression is involved in carcinogenesis and progression of cancer. Furthermore, Skp2 expression was also inversely correlated with PTEN expression and p27 expression was positively related with PTEN expression in gastric carcinoma. PTEN tumor suppressor acts as a phosphatase of phosphatidylinositol-3,4,5-trisphosphate (PIP3) and negatively controls the G_1/S cell cycle transition and regulates the levels of p27[29]. Mamillapalli *et al*.[21] showed that PTEN deficiency in mouse embryonic stem cells decreases p27 level while increases Skp2 level. Conversely, in human glioblastoma cells, ectopic PTEN expression leads to p27 accumulation accompanied with a reduction of Skp2 and ectopic expression of Skp2 alone is sufficient to reverse PTEN-induced p27 accumulation, restore the kinase activity of cyclin E/CDK2 and partially overcome the PTEN-induced G_1 cell cycle arrest. Recombinant SCF^{Skp2} complex or Skp2 protein alone can rescue the defect in p27 ubiquitination in extracts from cells treated with a PI 3-kinase inhibitor. Yang *et al*.[30] showed that loss of expression or reduced expression of PTEN protein contributes to carcinogenesis and progression of gastric carcinoma. Myung *et al*.[31] reported that reduced expression of cyclin-dependent kinase inhibitor p27 is associated with advanced stage and invasiveness of gastric carcinoma. Additionally, Yang *et al*.[22] also found that elevated Skp2 protein expression in human prostate cancer is associated with loss of p27 and PTEN expression. Thus, all these findings suggest that PTEN functions as a negative regulator of the Skp2 pathway that is normally used to control S-phase entry through the regulation of p27 in gastric carcinoma and the effects of Skp2, p27 and PTEN together play an important role in carcinogenesis and progression of gastric carcinoma. These results also suggest that Skp2 functions as a critical component in the PTEN/PI3-kinase pathway for the regulation of p27.

In conclusion, alterations in the levels of proteins controlled by the ubiquitin–proteasome pathway during transformation are likely to be common. Further studies that define how such posttranscriptional control pathways are altered during transformation may provide additional biomarkers or facilitate the identification of novel therapeutic targets. The finding that Skp2 is induced in a number of different cancers suggests that drugs directed at this molecule may provide a more selective target for therapeutic development.

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• *CASE REPORT* •

Regression of hepatocellular carcinoma during vitamin K administration

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Abstract

An 85-year-old man with HCV infection and diabetes mellitus was diagnosed as having hepatocellular carcinoma (HCC, 13 cm in diameter) based on high serum alpha-fetoprotein (AFP), AFP-L3, and des-γ-carboxy prothrombin levels as well as typical enhancement pattern on contrast-enhanced CT. The patient did not receive any interventional treatments because of advanced age and the advanced stage of HCC. He chose to take vitamin K, which was reported to suppress the growth of HCC *in vitro*. Three months after starting vitamin K, all three tumor markers were normalized and HCC was markedly regressed, showing no enhancement in the early arterial phase on CT. Here we present the report describing the regression of HCC during the administration of vitamin K.

Key words: Hepatocellular carcinoma; Vitamin K; Regression

Nouso K, Uematsu S, Shiraga K, Okamoto R, Harada R, Takayama S, Kawai W, Kimura S, Ueki T, Okano N, Nakagawa M, Mizuno M, Araki Y, Shiratori Y. Regression of hepatocellular carcinoma during vitamin K administration. *World J Gastroenterol* 2005; 11(42): 6722-6724
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INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common cancers worldwide[1]. HCC is developed mainly in patients with chronic hepatitis C or B virus infections and the screening of this high risk group makes it possible to detect HCC at the early stage. However, many patients are still diagnosed at the advanced stage and the prognosis is poor. Transcatheter chemoembolization or chemotherapies are performed in cases of advance HCC[1-3]. These treatments are sometimes very effective, but are harmful to the background liver and accelerate the progression of pre-existing chronic liver injury. Therefore, these therapies cannot be performed in patients with severe liver cirrhosis. In addition, severe side effects often impair the quality of life. Thus, the development of new low-invasive therapies is needed.

Vitamin K is a factor in blood coagulation and is used for the treatment of osteoporosis[4]. Vitamin K is also known to inhibit the growth of cancer cell lines, but its clinical usefulness in the treatment of human cancer remains controversial[5-7]. In this report, we present a case of advanced HCC in which marked regression during the administration of vitamin K has been documented.

CASE REPORT

In July 2003, an 85-year-old man with liver cirrhosis due to HCV and diabetes mellitus was admitted to our hospital because of giant liver tumors. The patient had no history of alcohol drinking or blood transfusion. He did not smoke and was not taking any medicine, but was under intermediate-acting insulin (12 U/d) injection. He had undergone surgery for prostate hypertrophy 3 years before the present admission. Routine laboratory studies showed Hb 13.8 g/L, platelet count 101 000/mm^3, albumin 3.2 g/L, total bilirubin 0.6 mg/dL, aspartate aminotransferase and alanine aminotransferase 232 U/L and 102 U/L respectively, γ-glutamyl transpeptidase 93 IU/L, fasting blood glucose 126 mg/dL, HbA1c 11.1%. Serum alpha-fetoprotein (AFP), lentil lectin-A-reactive AFP (AFP-L3), and des-γ-carboxy prothrombin (DCP) were 1 212 ng/mL, 76.9%, and 51 300 mAU/mL, respectively. The patient was positive for HCV antibody and hepatitis B virus core antigen but negative for hepatitis B virus surface antibody. There were no esophageal or gastric varices observed during gastro-intestinal endoscopy. Although the patient did not complain of abdominal pain, gastric ulcer was detected by endoscopy and administration of the proton pump inhibitor was started.

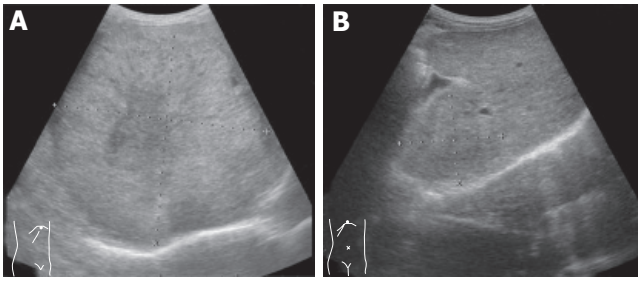

Figure 1 Giant tumor with central hypoechoic area dominated the right lobe of the liver (**A**) and remarkable regression of tumor with obscure margin after administration of vitamin K (**B**).

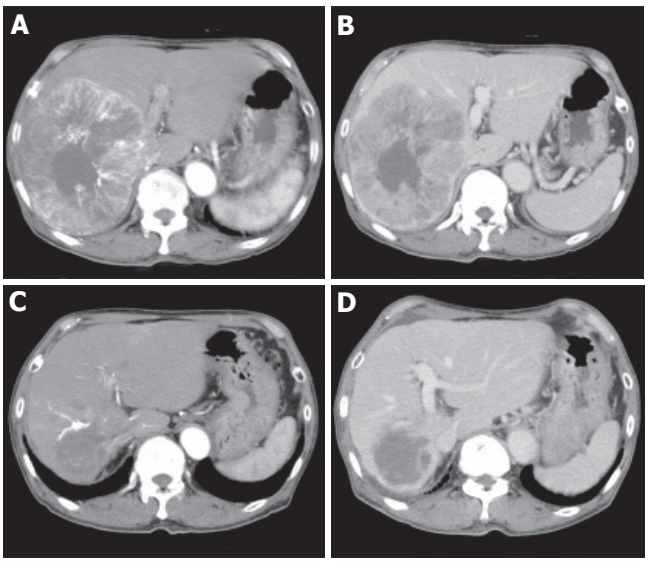

Figure 2 Enhancement of peripheral portion of tumor during early arterial phase (**A**) and hypoattenuation during portal phase (**B**) as well as remarkable tumor regression and no enhancement during early arterial phase (**C**) and portal phase (**D**) 5 mo after administration of vitamin K.

Abdominal ultrasonography (US) demonstrated that a large hyperechoic mass (13 cm in diameter) with central hypoechoic area dominated the right lobe of the liver (Figure 1A). At least four nodules about 2 cm in diameter surrounded the main nodule and another nodule (5 cm in diameter) existed at segment 4. Portal vein (PV) in the right lobe was replaced by tumor except for the PV in segments 5 and 8 in which faint blood flow was observed. Right hepatic vein was replaced and the middle hepatic vein was stretched by the tumor. Enhancement during the early arterial phase and defect during portal phase were observed by contrast enhanced-computed tomography (CE-CT, Figures 2A and B). Based on typical imaging features and elevated HCC tumor markers, the liver tumors were diagnosed as HCC. There was no distant metastasis observed by chest X-ray or abdominal CT.

Because of the patient's advanced age and advanced stage of HCC, the patient declined interventional therapies or angiography. Thereafter, we prescribed oral vitamin K_2 (45 mg daily), which was reported to inhibit cancer growth

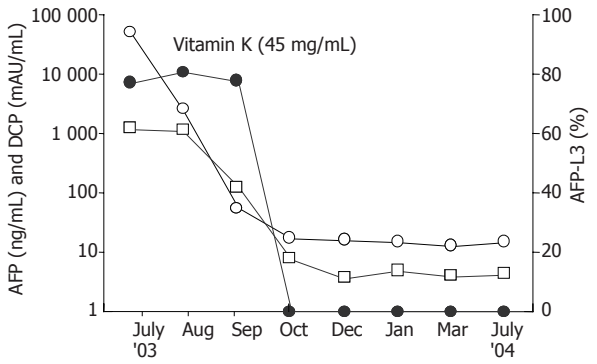

Figure 3 Clinical course of the patient.

in vitro[4,5]. The recommended dose for osteoporosis therapy was used. Informed consent for the administration of vitamin K was obtained from the patient and his family. Soon after the administration of vitamin K, AFP as well as DCP decreased and finally normalized in the 3rd month. AFP-L3 also decreased to below the detectable level from 76.9% to 0% (Figure 3). CE-CT, 5 months after starting vitamin K, demonstrated that the tumor sizes were remarkably decreased and the diameter of the main tumor was 5.5 cm (Figures 2C and D). Enhancement during the early arterial phase had disappeared in all nodules. US demonstrated that the tumor regressed and the margin of the tumor became obscure (Figure 1B). Twenty-two months have passed and there are no signs of recurrence observed to date. All three tumor markers have remained within the normal limits.

DISCUSSION

Vitamin K is known to inhibit the growth of many cancer cell lines including HCC[5–7]. The mechanisms of the anticancer action of vitamin K are not precisely understood, but several candidates including the induction of apoptosis, differentiation, and cell cycle inhibition have been proposed[4]. Recent research has demonstrated that the anticancer action of vitamin K may occur at the level of tyrosine kinases and phosphatase, modulating various transcription factors. In addition to the *in vitro* analysis, there are several case reports that described the usefulness of vitamin K for the treatment of human cancers including myelodysplastic syndrome (MDS)[6,7]. In a clinical trial of vitamin K for MDS and post-MDS acute myeloid leukemia, hematological improvement is reported in four of nine patients[8]. Recently, Koike *et al.* demonstrated that the development of PV tumor thrombus is inhibited and patient's survival is improved in patients with HCC following administration of vitamin K (unpublished data). These reports suggest that vitamin K is an anticancer agent for HCC.

Spontaneous regression of cancer is rare and estimated to occur once in 60 000-100 000 patients with cancer[9]. At least 27 cases of spontaneous regression of HCC have been reported[10-14]. There were no apparent clinical

characteristics discriminating the patient with spontaneous regression of HCC from others and various causes of regression have been documented (e.g. lack of blood supply due to arterial infarction or rapid growth of HCC, withdrawal from steroids, abstinence of alcohol consumption, administration of herbal medicines, and immunological activation due to high fever or sepsis)[10,15]. Hepatic angiography was performed in some cases and blood shortage due to the intimal damage induced by micro catheters could also be the cause of regression. In this report, the speed of tumor growth was uncertain because the patient was not followed up before admission. Other than that, he did not have any of the background factors reported previously. Therefore, possible reasons for regression are limited in this case; spontaneous regression due to blood shortage induced by rapid tumor growth or regression due to vitamin K administration, which was reported to inhibit tumor growth.

Although we could not conclude that administration of vitamin K was the cause of the regression in this case, this is a case report documenting regression of HCC during the administration of vitamin K. Further prospective study is needed to confirm the efficacy of vitamin K for the treatment of HCC.

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• *CASE REPORT* •

Unicentric Castleman's disease of the pancreas with massive central calcification

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Abstract

Unicentric Castleman's disease of the pancreas is extremely rare, with only six cases described in the worldwide literature. An asymptomatic case of unicentric, hyaline, vascular-type Castleman's disease (UCD) localized to the tail of the pancreas with central calcification imitating a primary neoplasm of the pancreas is presented. This is the first description of endosonographic and endoscopic retrograde pancreatographic findings of pancreatic UCD. Additionally, computed tomography, histological and serologic findings are reported.

Key words: Unicentric Castleman's disease; Calcification; Pancreas; Endoscopic ultrasound

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INTRODUCTION

Unicentric Castleman's disease (UCD), also called giant lymph node hyperplasia, follicular lymphoreticuloma or angiofollicular mediastinal lymph node hyperplasia, is a rare lymphoproliferative disorder of unknown aetiology. UCD was first described and defined by Benjamin Castleman in 1956 in tumors of the thymus with asymptomatic mediastinal lymph node hyperplasia, which could be separated histologically from thymomas and other neoplastic lymph node disorders[1]. Flendrig and Schillings distinguished two basic pathologic types and one mixed variant[2], while Keller *et al.* designated hyaline-vascular, plasma cell, and hyaline-vascular plasma cell type[3]. UCD typically presents as indolent benign disease in young adults with a median age of approximately 35 years and is equally distributed between males and females[2,3]. A subset of patients with plasma cell variant may have systemic symptoms. UCD in the abdomen and pelvis has been only described in the radiology literature as a focal enhancing mass of varying locations, including the retroperitoneum, mesentery, and hepatic porta[4].

CASE REPORT

A 53-year-old man was well until one week before admission with renal colic with an onset of pain strongest at the right paravertebral region and radiating in the right iliac crest. Excretion urography performed in another hospital showed a slight pyelectasis of the right kidney and a left renal duplication with ureter bifidus, while, incidentally, abdominal ultrasound showed a calcified mass (3.4 cm × 3.4 cm) in the left hilar region of the kidney. Then the patient was subsequently referred to our tertiary care centre for further evaluation and treatment .

On physical examination the patient was afebrile and appeared well. The pulse was 80 bpm and the blood pressure 130/70 mm Hg. No lymphadenopathy was found. A non-tender liver edge descended 2 to 3 cm below the right costal margin; the spleen was not felt. Peripheral blood counts, liver function tests, cancer antigen (CA 19-9), carcinoembryonic antigen (CEA) and β-2 microglobulin were within normal levels. Immunologic tests for hepatitis A, B, C were negative. An ELISA for human immunodeficiency virus antibodies was negative. A postoperative ELISA (assay against viral latency-associated nuclear antigen) and a polymerase-chain-reaction test for herpes simplex virus 8 was negative and the serum interleukin 6 level was normal.

The patient had undergone a thorough systematic investigation, which included abdominal ultrasonography, computed tomography (CT), endoscopic ultrasound with fine-needle aspiration and endoscopic retrograde pancreatography (ERP). A spiral abdominal CT examination showed a well-circumscribed (5.5 cm × 4.5 cm × 4.5 cm) hypervascularized mass of soft tissue density with focal calcification in the tail of the pancreas without evidence of invasion of surrounding structures or vessels (Figure 1A). A thoracic and neck CT was normal. Endoscopic ultrasound revealed a solid, well defined, slightly heterogeneous, hypoechogenic soft-tissue mass with central calcification. Local lymph nodes were not enlarged (Figure 1B). The cytological evaluation of

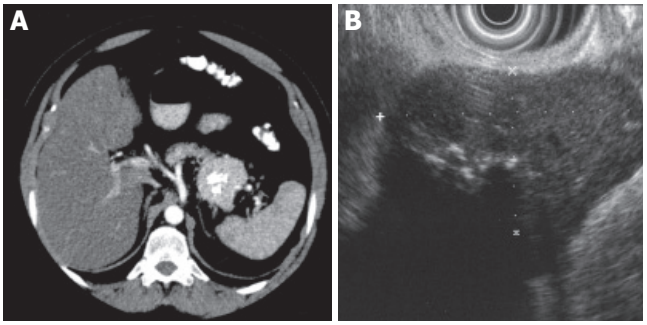

Figure 1 A: Enhanced CT shows a well-circumscribed hypervascular mass of soft tissue density with focal calcification in the tail of pancreas; **B:** 7.5 MHz endosonography shows a well-circumscribed hypoechogenic mass (2.8 x 4.9 cm) with focal hyperechogenicity and without signs of infiltration.

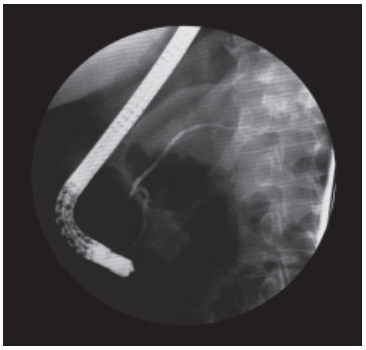

Figure 2 Endoscopic retrograde pancreatography (ERP) shows no malignant stricture of the pancreatic duct and a slight parenchymography in the tail of the pancreas.

endosonography controlled fine-needle aspiration biopsy disclosed lymphatic tissue with focal sclerosis. In ERP (Figure 2) a malignant stricture of the pancreatic duct could be ruled out and a slight parenchymography in the tail of the pancreas with circular calcification of the tumor projecting around the pancreatic duct was shown. Postoperative microscopical examination of a specimen of bone marrow obtained by needle aspiration biopsy disclosed only slight hypercellularity.

Then the patient subsequently underwent left hemipancreatectomy with splenectomy. The specimen of hemipancreatectomy had a spheroidal tumor mass measuring up to 4.6 cm, which was demarcated by a fibrous pseudocapsule (partially with tumor infiltration) from normal tumor-free pancreas tail tissue (12 cm × 7 cm × 4 cm, 367 g). Remarkably, there was a central calcification 3.2 cm in diameter (Figures 1A and 3B). Microscopic examination of the mass revealed germinal centres *with hyalinized venules* originating from a hypervascular mantle zone surrounded by small lymphocytes in a concentric onionskin pattern with focal hyaline deposits, which is characteristic for hyaline-vascular type of Castleman's disease (Figures 3A and 3B). The interfollicular stroma showed few plasma cells expressing light chains and IgM. The patient's

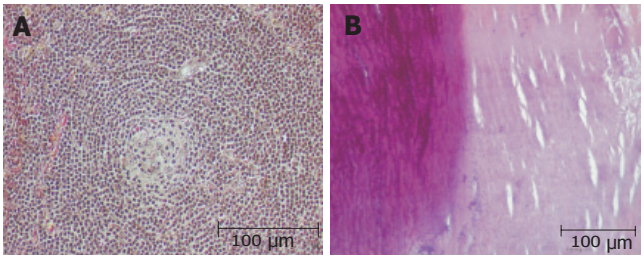

Figure 3 A: Germinal centers with hyalinized venules originating from a hypervascular mantle zone surrounded by small lymphocytes in a concentric onionskin pattern (hematoxylin and eosin); **B:** Areas of heterotopic calcification of the central part of the tumor (left panel) and hyaline deposits (right panel) (hematoxylin and eosin).

postoperative course was without complications and no recurrence has occurred after a two year follow up period in our patient.

DISCUSSION

Unicentric Castleman's disease commonly occurs in the mediastinum (over 50 %), but has also been identified in multiple anatomic locations, including the neck, the pelvis and the axilla[5]. So far, UCD in the abdomen and pelvis has been described in the radiology literature as a focal enhancing mass of varying location, whereas UCD in the pancreas constitutes an extremely rare entity, which can imitate a tumor originating from pancreatic tissue[4]. Prior to this report, endoscopic retrograde cholangiopancreatography and endoscopic ultrasound findings of pancreatic Castleman's are not available. The patient had no systemic symptoms, the pancreatic mass was well defined and hypoechogenic on endoscopic ultrasonography and showed no contact between the main pancreatic duct with circular calcification projecting around the pancreatic tail at ERP. On abdominal CT homogeneous enhancement with central pattern of calcification was found, as described in the literature[4,6,7]. Hence, possible differential diagnoses of this indeterminate tumor of the pancreatic tail containing central calcifications were acinar cell carcinoma, solid and papillary epithelial neoplasm, solid cystic tumor, serous cystadenoma or cystic teratomas of the pancreas, which are all known as well defined tumors with calcification patterns[8,9]. Castleman's disease in the abdomen and pelvis displays a variety of calcification patterns, including punctuate, coarse, peripheral, and "arborizing". In a recently published retrospective review of 16 cases of abdominal and pelvic manifestations, 31% showed calcification, one with an arborizing pattern and the remaining lesions with punctuate calcification[4]. Calcification with a diameter of 3.2 cm in a case of monocentric or multicentric disease has as yet not been observed.

Patients with the hyaline-vascular subtype are usually clinical asymptomatic, as was our patient. Only 3% of the patients present with systemic complaints[3] and less

than 10% (usually patients with plasma cell variant) have disease associated syndromes like myasthenia gravis, erythema nodosum, Horton's arteritis or Moschcowitz' s disease[5,10-13]. In contrast, the multicentric type of Castleman disease (MCD) is accompanied by a general lymphadenopathy, systemic symptoms, laboratory abnormalities, organomegaly and progressive clinical course with potential for malignancy and was not recognized until 1978[14]. Understanding of MCD has greatly expanded since the identification of its association with HIV and HHV-8 infection[15-17]. However, the sources of the immune activation in the unicentric form and the HHV-8 and HIV negative multicentric form are still unidentified. In retrospective studies, complete surgical resection for patients with unicentric hyaline vascular Castleman's disease has been associated with the best chance of cure [18].

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• *CASE REPORTS* •

Bile peritonitis due to intra-hepatic bile duct rupture

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Abstract

Generalized biliary peritonitis is a serious intra-abdominal emergency. Most of them occur due to duodenal ulcer perforation and rapidly evolve into bacterial peritonitis due to contamination by gut organisms and food. In this situation, recognition of the pathology and its treatment is straightforward and is usually associated with a good outcome. There are a few unusual causes of biliary peritonitis, of which rupture of the biliary tree is one. We describe a rare case of biliary peritonitis due to rupture of an intra-hepatic biliary radical. Unusual causes of peritonitis do interrupt our daily routine emergency surgical experience. Rapid recognition of the presence of peritonitis, adequate resuscitation, recognition of operative findings, establishment of biliary anatomy, and performance of a meticulous surgical procedure resulted in a good outcome.

Key words: Biliary peritonitis; Intra-hepatic biliary radicle; Rupture

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INTRODUCTION

Rupture of an intra-hepatic biliary duct leading to biliary peritonitis is a rare occurrence, with only few cases reported in the literature[1-4]. This case report reinforces the necessity of complete and meticulous operative assessment of the biliary system in every case of bile peritonitis.

CASE REPORT

A 78-year-old male with severe spondyloarthropathy presented with a 12-h history of upper abdominal pain, nausea and vomiting. He was shocked (BP 80/40, pulse 120/min) and clinical examination revealed guarding, tenderness with rebound in the upper abdomen and absence of bowel sounds. A diagnosis of peritonitis was made and he was appropriately resuscitated. He responded well to the treatment and became hemodynamically stable. His hematological blood profile as well as his urea and electrolytes were unremarkable. He had conjugated hyperbilirubinemia with raised alkaline phosphatase and alanine transaminase. Plain radiology (erect CXR and abdominal films) did not demonstrate any pneumoperitoneum.

When the patient was optimized, he underwent surgery. At laparotomy, there was no evidence of gastrointestinal perforation; however, free intra-peritoneal bile was found. The extra hepatic biliary system was grossly dilated due to obvious obstruction at the distal common bile duct by an impacted calculus. The gall bladder contained calculi and was thick walled. There was a perforated superficial biliary radicle in the left lobe of the liver, which was the source of the free bile in the peritoneal cavity.

A cholecystectomy was performed and the CBD was explored. The calculus in the distal CBD needed to be extracted through a trans-duodenal sphincterotomy. Post exploratory choledochoscopy and on-table cholangiography confirmed clearance of the bile ducts. The common bile duct was drained with a T-tube and at the end of the operation the ruptured biliary radicle stopped leaking bile. It was however reinforced with two interrupted 3/0 prolene sutures. The right sub-hepatic space was drained and the abdomen was closed as per standard.

The patient was transferred to the high dependency unit for immediate care. He made a slow, but steady post-operative recovery. T-tube cholangiogram performed on day 14 (post-op) confirmed free passage of contrast into the duodenum and absence of residual CBD stones; thus allowing its removal. The patient was discharged 34 days following surgery and he is still healthy with normal liver function tests when last reviewed 2 years following surgery.

DISCUSSION

Peritonitis requiring surgical intervention is caused by perforated peptic ulcer in about 40% cases (duodenum: gastric: 3:1), appendicitis in 20%, gangrene of the small bowel or gall bladder in 15%, post-operative complications in 10% and miscellaneous causes in 15% cases[5]. Most commonly, peritonitis in the clinical setting is due to microorganisms, though the initial insult is usually chemical as in peptic ulcer perforation where bile, pancreatic

enzymes, blood, etc., gain access into the peritoneal cavity.

The peritoneum can be contaminated with bile through a number of routes. The commonest is post-cholecystectomy. This is usually due to the division of small bile channels between the gall bladder and liver, imperfect clipping of the cystic duct, residual CBD stones causing raised intrabiliary pressure and inadvertent division of an accessory hepatic duct. The latter is potentially serious usually requiring biliary reconstruction. Other causes include post liver transplant biliary peritonitis, spontaneous hepatic rupture in pregnancy and trauma to the extra-hepatic biliary system such as that following minimal access renal surgery[6].

Perforation of the biliary tract secondary to rupture of the gall bladder (empyema/gangrene) is well documented. However, spontaneous rupture of the CBD is exceedingly rare and here the etiologies are increased intra-ductal pressure, calculus erosion and necrosis of the duct wall secondary to thrombosis[7]. Spontaneous perforation of extra hepatic ducts is also a very rare cause of jaundice in infancy[8]. The commonest site is the confluence of the cystic and common hepatic ducts[9]. Biliary peritonitis secondary to intra-hepatic duct rupture is rarely reported in the literature; the causes are calculus disease of the biliary ducts (as in this case), stenosis of the papilla and Caroli's disease[10].

The clinical picture associated with biliary peritonitis varies and the correct pre-operative diagnosis is difficult. This combined with the associated comorbidity of the patient population (mainly elderly) significantly contributes to a mortality rate of 30–50%[11]. Though the initial insult by bile is chemical, secondary bacterial infection is the usual sequelae. Furthermore, it has been clearly shown that, in the presence of bacteria, bile further impairs local host defense mechanism through its detergent lytic effects[12]. Paralytic ileus is also a frequent complication. Laboratory findings are usually non-contributory but biliary peritonitis should always be suspected in any patient with unexplained abdominal symptoms.

The aim of treatment is to prevent sepsis in the abdominal cavity and thus prompt recognition of the condition and control of source of the contamination with appropriate drainage/reconstruction of the biliary system is of paramount importance. The type of surgery is dependent upon the general condition of the patient as well as on biliary anatomy. Regardless, biliary peritonitis requires some form of drainage, either externally via the percutaneous route or internally via the endoscopic/open surgery route. In our patient, the intra-hepatic duct rupture was presumably due to a very high pressure in the biliary system secondary to calculus obstruction in the distal common bile duct. Transduodenal sphincterotomy and extraction of the impacted calculus along with 'T' tube drainage resulted in an uneventful resolution. Prompt recognition of this condition before biliary/systemic sepsis supervened played a major role in the positive outcome for this patient. In conclusion, prompt laparotomy in a well-resuscitated patient and an adequately tailored operation depending on the operative findings are the mainstay to avoid local and systemic sepsis and long-term morbidity in these cases of peritonitis.

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