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World Journal of Gastroenterology (*World J Gastroenterol , WJG*), a leading international journal in gastroenterology and hepatology, has an established reputation for publishing first class research on esophageal cancer, gastric cancer, liver cancer, viral hepatitis, colorectal cancer, and *H pylori* infection, providing a forum for both clinicians and scientists, and has been indexed and abstracted in Current Contents®/Clinical Medicine, Science Citation Index Expanded (also known as SciSearch®) and Journal Citation Reports/Science Edition, *Index Medicus*, MEDLINE and PubMed, Chemical Abstracts, EMBASE/Excerpta Medica, Abstracts Journals, *Nature Clinical Practice Gastroenterology and Hepatology*, CAB Abstracts and Global Health. ISI JCR 2003-2000 IF: 3.318, 2.532, 1.445 and 0.993. *WJG* is a weekly journal published by The *WJG* Press. The publication date is on 7th, 14th, 21st, and 28th every month. The *WJG* is supported by The National Natural Science Foundation of China, No. 30224801 and No.30424812, which was founded with a name of *China National Journal of New Gastroenterology* on October 1,1995, and renamed as *WJG* on January 25, 1998.

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LEADING ARTICLE

How to establish a first-class international scientific journal in China?

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Abstract

Hundreds of scientific journals are published in China. However, only scores of them are included in Science Citation Index by the Institute for Scientific Information, with impact factors of only 1 or less. Thus, how to establish a first-class international scientific journal in China is an important but difficult topic that deserves extensive exploration. *World Journal of Gastroenterology* (*WJG*) sets a good example although it has experienced setbacks on the road towards success. Concepts and pursuits that affirm the overall development direction, innovation and dreams that provide impetus and aspiration for higher objectives, team work and unique pattern that assure excellent quality and service, and culture and environment that also determine the speed and direction of the development, are believed to be the major factors contributing to the success of *WJG*. It is recommended that the effective resolution to the above issue is to learn from Chinese examples such as *WJG* rather than from "how foreign journals do".

Key words: First-class scientific journal; Concepts and pursuits; Innovation and dreams; Team work and unique pattern; Culture and environment; *World Journal of Gastroenterology*

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INTRODUCTION

In 2005, China Ministry of Science and Technology organized and implemented a research project on "How to establish a first-class international scientific journal in China". This issue is indeed an important but difficult topic that deserves extensive exploration. Both the editorial workers and the administrative authorities of scientific journals should seriously respond to it. In the meantime, I believe that the effective resolution to this issue is to learn from "Chinese cases"rather than from "how foreign journals do".

World Journal of Gastroenterology (*WJG*) was formally established by Dr. Lian-Sheng Ma on October 1, 1995. As an administrative leader of the Science Committee of Shanxi Province which is in charge of scientific journals, I have witnessed the growth and development of *WJG* over the past 12 years, but never made any public comments on the journal. Today, I wish to make some points by incorporating the *WJG* case into the above mentioned topic.

CONCEPTS AND PURSUITS

As a macroeconomist, I know nothing about scientific journals. However, I am astonished to find that many journals have the same weaknesses as enterprises do. Some experts have reported that many Chinese enterprises suffer from "short-life syndrome" with an average lifespan of 2.9 years. It has also been said that some large Chinese corporations suffer from "small enterprise disease" characterized by severe opportunism in the strategic plan, lack of supporting sections in organization structure, collaboration inside the teams, and the uniform core values of the corporation. Similarly, it has been said that Chinese scientific journals are in short of creative and innovative papers, and abundant in third-class and "cloned" papers, which not only is a great waste of academic resources but also results in an extremely bad impact on scientific development in China. In my opinion, Chinese scientific journals share one common weakness, i.e. lack of concept and pursuit. This is just like the situation in Shanxi Province, where the negative impact driven by so-called resource economy has become increasingly obvious. Unauthorized mining, frequent mine explosion, vicious competition and corruption all can be attributed to the lack of concepts. Shanxi economy is an example that is cursed by "its resources". The misfortune resulting from the lack of concepts is shown by the horrible economic development without any conceptual restriction. Similarly, the development of scientific journals without any conceptual restriction is disastrous since it brings about enormous scientific "rubbish" and academic corruption,

which is known as "concept-lacking syndrome"in China.

Concepts mean belief, pursuits, and direction. Its core is the concept of values. The world well-known United States Military Academy at West Point has a history of over 200 years. The Academy's special educational concepts are "nation, honor and responsibility", which represent the spirit of nation, honor for society, team and personal responsibility. With such concepts, the Academy has educated two presidents (Ulysses S. Grant and Dwight D. Eisenhower), many famous generals, and a lot of business leaders. Whether an entrepreneur pursues a career or profits is the criterion to judge his/her personality. What is the objective to establish an academic journal? I said to Dr. Ma 10 years ago that "You should not be involved in any academic journal if you just want to make money". He got what I meant very clearly. Frankly, I did not believe he was able to establish an academic journal at that time because he lacked resources. Moreover, I did not understand his intention or ambition. On the one hand, he was already a famous doctor, and made a lot of money from his herbal inventions at that time, and thus could live a very comfortable life. On the other hand, it requires quantities of human and financial resources to take many risks to establish an academic journal. To choose establishing an academic journal as a career means hard work, suffering and even tears.

With high quality and excellent service as his concepts, Dr. Ma has devoted himself to and sacrificed a lot for *WJG*. As a man in his forties, his hair has turned grey. Fortunately, he finally succeeded. In the article entitled "The reasons for *WJG*'s success", which describes that the ultimate criterion to test a scientific journal is whether or not researchers in the field put it in their reading list. *WJG* has now successfully passed the criterion. *WJG* is an open and free international journal, and also the only weekly journal in gastroenterology in the world. The latest published papers in *WJG* are always sent to doctors and researchers free of charge by email. In addition, an average of about 30 000 online visits to the *WJG* website is recorded every day.

WJG has proposed a higher objective. At present 47 journals in Gastroenterology worldwide are included in the Science Citation Index Expanded (SCI-E). These first-class journals are also classified into three grades. Dr. Ma plans to invest one hundred million Yuan to make *WJG* an upper-middle level journal among these journals within 5 to 10 years. He is exploring a unique way to establish an international first-class scientific journal in China with persistent pursuits for quality and services.

INNOVATION AND DREAM

Almost all the industries find it hard to adapt to the new environment in which there is a changing process from an industrialized society to a knowledgeable society, and do not know what to do next. I attended a recent forum on education reform during which a Japanese expert introduced education reform in Japan. "The most lacking nowadays are concepts and ideas" he said emotionally. It is really difficult to depict the pattern or model of scientific journals in the future. It was reported in a survey that only less than 5% Chinese enterprises have made a professionally-standard, complete and feasible strategicaly developed plan by scientific means. More than 90% of companies have no long term plan on how to survive. There is a famous byword at Harvard University, that is, a successful man is distinguished not due to his knowledge and experience, but due to his way of thinking. It has been said that the future cannot be forecasted or adapted, but can only be created. What is a first-class international scientific journal in China? This question can only be thought and answered by ourselves. Creativity is always limited by knowledge, experience and way of thinking. As a president of a publisher or an editor-in-chief of a journal, one must have the specific ability to shape innovative thinking, and to improve his thinking to a higher level.

Over the past decade, the most that Dr. Ma and I have discussed is the creativity of *WJG*. Dr. Ma telephoned me almost every week after my retirement, telling me his new ideas, from the administrative system to the flow remodeling, from scientific editing to electronic editing, from human resources to the internal structure management, and from exiting journals to resource integration in the same field. He is pursuing innovation in every aspect of editing a journal, which is the major reason that I have been greatly interested in *WJG*. On January 9, 2006, President Jin-Tao Hu pointed out at the National Congress of Science and Technology that China shall become a country with innovation in the next 15 years. This is a new starting point to a new road. Now officials in every industry are thinking about the issue on how to exploit a path with independence, innovation and Chinese characteristics. Then, how to establish an international first-class scientific journal in China? Taking the laggard reality in China into consideration, I would suggest that innovation must be comprehensive and systematic, rather than individualized, revolutionary, and evolving, in order to catch up or even overtake the international levels. Moreover, innovation in the administrative system is much more important than innovation in technology. I believe that one can find all answers to the question if he/she sticks to the two principles of innovation.

Now *WJG* Press, based on the current achievements and experience, is building an innovative system that includes journals as the main body, and the laboratory and pharmaceutical companies as supporting points, and is exploring a new model that combines journal publication with manufacturing, education and research. Dr. Ma always tries hard to make the best *WJG* once he identifies the right way, with the strategic design and practical implementation being carried on almost at the same time. *WJG* was publicly issued on June 29, 1995 as a seasonal journal. It became bimonthly from 1998 to 2002, monthly in 2003, biweekly in 2004, and then weekly in 2005, with one big jump each year over the last three years. Despite such significant achievements, I described each jump as the "thrilling jump" (Karl Max). I have warned Dr. Ma that if the jumping failed, the injured would be Dr. Ma himself but not *WJG*. Fortunately, Dr. Ma succeeded. Now he is thinking how to integrate resources of the famous journals in the field, and is planning to publish *World Journal of Oncology* and *World Journal of Chinese Medicine*, and to create a

broader platform for medical communications. In this way, an innovative chain will be created to optimize resource allocation, just like the supply chain management in the enterprises. I believe that he will succeed again this time since the innovation will not be limited in one journal or one step, and thus will improve the whole academic level.

TEAM AND PATTERN

Only a first-class team can publish first-class journals. The *WJG* editorial board is composed of 760 experts in gastroenterology from 52 countries. Many famous scientists have been invited to write reviews and editorials for *WJG*. Its permanent editorial team is composed of those outstanding staff with doctors, masters of bachelor degree, and has become more competitive and efficient.

There were some bad comments at the beginning of *WJG* when there were not many resources such as financial support for journals from the government and experienced editors. There was a doubt on the success of *WJG* that was run by an "individual". Sometimes, I wonder how many emergent novelties have been killed by such an attitude and way of thinking. Everything has its own developing and improving process, which is from small to large and from weak to strong. The Editor-in-Chief is the key to a journal, just like an entrepreneur to an enterprise. A recent book "The World Is Flat" by an American writer, Thomas L. Friedman, describes that the world has become smaller and smaller. In the opinion of the author, the world has experienced three "shrinking" processes. The first one took place in the fourteenth century, and is characterized by the expansion of countries. At that time, Columbus discovered the New Continent, which opened the sea route and thus reduced the distances between countries. The second one took place in the industrial revolution, and is characterized by the expansion of enterprises (multinational corporations). At that time, the development and production of the railway, electricity and telecommunication instruments further reduced the size of the world. The third one is taking place now following the significant development of information network, and is characterized by the expansion of personal power. I heard recently that there is a news agency in the United States run by a single person. Therefore, we should not underestimate or ignore the ability of any individuals. To some extent, even an individual is able to influence the whole world.

Composition of a team is always influenced by the strategic orientation and the tactical policy, and restricted by the administration system and operation mechanisms. *WJG* has two principles. One principle is to operate according to the international standards. Dr. Ma has described to us the general situation of international standards and market operation patterns in 15 aspects, such as administrative system, editing criteria, paper quality, online submission, invitations for editorials and reviews on highlighted topics, peer review, external communications, printing and distribution, *etc*. This year, 75.8% of manuscripts submitted to *WJG* were from authors outside of China. The other principle is to improve with informatization. In some senses, internationalization means internet communications. *WJG* always improves continuously its business flow in order to match its administration pattern with informatization. I read a worksheet about the editing process of *WJG*, which is composed of 29 steps for scientific editing, 36 steps for electronic editing, and more than 10 other basic steps. Scientific and strict regulations and operational patterns are the basis of a successful *WJG*. The quality of a journal must be improved with time, and its development must represent the changing world. Unfortunately, some journals are sill run with the management pattern designed for the outdated planned economy. These journals lack in vitality and compatibility despite abundant human resources and financial support. Now is the time when live fish eat dead fish, and fast fish eat slow fish, but not necessarily big fish eat small fish. This is due to the choice of the market rather than the instruction of government. I used to recommend the pattern of *WJG* to some of my friends in the field. They all made good comments on the pattern but could not learn from it very well. Structure and administrative pattern are determined by the system. Therefore, it is the issue of the system that we must resolve before we find an international first-class scientific journal in China.

CULTURE AND ENVIRONMENT

A journal, or an enterprise or a university, or an institute, or even a development zone can only grow in a certain culture and environment. They are all the products of the culture and environment. Why is the United States of America so strong? Is it just because of its military power and technology? No, it is because of its culture based on its democratic construction and citizen quality. Michael Bott, a famous American scholar has pointed out that the advantage that is based on culture is the most difficult to copy or replace, the most fundamental, the most everlasting and thus the most pivotal advantage in competition. China is investigating how to develop the "middle part" these days. Some experts believe that the key to developing the "middle part" is reshaping of the local culture. What I learned from the growing process of *WJG* is that some aspects of our culture really need improvement. Some government officials created difficulties for *WJG*, and some authorities turned their noses at *WJG*. In addition, some "readers" abused *WJG* or even insulted the publisher with whatever words they could create on the internet, such as"*WJG* is a bad journal", "I feel shamed that my paper is published in *WJG*". What kind of culture is it where you cannot do anything? Who are the victims of such a culture? We must have courage to reflect and animadvert on the outdated culture, and investigate these details in depth in order to found an international first-class scientific journal. One may get some insight from the book "Details Determine the Success".

The culture in the Silicon Valley or the Tech-Garden of Zhongguan Village is non-traditional. Individual behavior is determined by the profits and dreams. There is no authority or god. There are only heroes from commonality. So Silicon Valley is the place for youngsters to begin their career, to carve out, and to realize their dreams. Persons in Silicon Valley always respect losers, because they know

that the losers have taken the risks and paved the path for the victors towards the hi-tech peak. Mistakes and failures are unavoidable in its growing process. *WJG* was refused to be included in SCI in 2004 and 2005, which was a big blow to Dr. Ma. He made intensive investigations, and found out the reasons. The *WJG* self citation rate of 94%, which was rather high and made its impact factor up to 2.532 in 2002, was the major reason for the refusal. High impact factor and compositor were usually induced by high self citation rate, which resulted in a misleading of the influence of *WJG* in the field. Dr. Ma reduced its self citation rate to 15.87% in 2005, and fortunately *WJG* recovered by SCI this year. This is probably a good lesson. In addition to Dr. Ma's hard work, the success of *WJG* is also attributed to the support from some related authorities, such as China Ministry of Science and Technology, National Administration of News and Publication, Natural Science Foundation Committee of Shanxi Province, and many experts and readers. Professor Fa-Zu Qiu, member of the Chinese Academy of Sciences, aged 93, and Mr. Xu-E Li, former vice-director of China National Science and Technology Committee, both gave *WJG* tremendous support. Thus, *WJG* is very fortunate to have such support. We must learn from the growing process of the international first-class scientific journals if we want to found such a journal. The processes are always more splendid than results. Every time when *WJG* steps forward, I would remind Dr. Ma that happiness of success is temporary while the suffering and challenging are everlasting. Indeed, the most valuable personality of modern entrepreneurs is willingness to pursue "suffering" and challenge.

I have studied a book "Laws Cannot Change the Society: How to Reform France", written by a famous French sociologist, Michel Crozier. In his opinion, with the development of society, communication between individuals becomes more and more complicated, the society becomes more and more fragile, and thus the outdated social administrative systems become weaker and weaker and the laws can no longer resolve the social problems. The impetus of the society is the human resources, especially those intellectuals with great potentials. Only by motivating their enthusiasm, can the social reform be performed. Generally, those who are engaged in scientific journals are the nation's backbone.

In April 2006, *WJG* held the first working meeting of the year. I was initially invited to give a lecture on the relation between morality and quality, but I was unable to attend the meeting. Later, Dr. Ma told me an interesting story. One day he attended lectures on two topics delivered by Professor Wu-Zong Zhou from University of St. Andrews, St. Andrews, the United Kingdom. The first topic was "Why to Publish Scientific Papers", and the second one was "Strict Academician Attitude Ensures the Quality of Scientific Papers". During the lecture, someone raised a comment, saying "we are coming here to learn how to write papers, not to accept moral education". Surprisingly, about two thirds of the audience applauded for the comment. Professor Zhou shook his head and could not say anything. So did I after I heard the story. There is an extensive debate on the comparison of the development between China and India in recent two years. Some people believe that intellectual community which represents the advanced productivity is better qualified in India than in China. The former American President Bill Clinton also holds this viewpoint. Last year, I read an article by Dr. Nan-Ping Yuan, the consul general of the Chinese Consulate in Bombay, India. The title was "What Is stronger in India than in China?". It describes that Indian intellectuals are determined to retain their independent personality with consistency and conscience. They do not compromise to obtain any benefits. They sympathize and help the weak. They possess excellent academic quality, without flippancy and the urge for a quick success and an instant benefit. They rarely have the concepts such as "job-hopping" and "becoming official and making money" in mind. I believe that all these characteristics that Indian intellectuals possess are the core compatibility that a journal or a region requires.

WJG has represented China to compete on the international academic stage without any national financial support. Many experts and scientists in Western countries praise this kind of intellectual such as Dr. Ma as "scientific fighters" and "academic faeries". At least, we should show our sympathy and respect to those who dedicate themselves to their national academy.

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EDITORIAL

Current concepts and controversies in the treatment of alcoholic hepatitis

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Abstract

The treatment of alcoholic hepatitis remains one of the most debated topics in medicine and a field of continued research. In this review, we discuss the evolution of scoring systems, including the recent development of the Glasgow alcoholic hepatitis score, role of liver biopsy and current treatment interventions. Studies of treatment interventions with glucocorticoids, pentoxifylline, infliximab, s-adenosyl-methionine, and colchicine are reviewed with discussion on quality. Glucocorticoids currently remain the mainstay of treatment for severe alcoholic hepatitis.

Key words: Alcoholic hepatitis; Treatment; Glucocorticoid; Biopsy; Scoring system

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INTRODUCTION

The treatment of alcoholic hepatitis is one of the most debated topics in medicine. The prevalence of the disease, its high fatality rate, and the elusiveness of cure keeps this disease in the forefront of topic reviews and scientific investigations.

Alcoholic liver disease accounts for over 12 000 deaths per year and 300 000 years of potential life lost in the United States[1]. Age adjusted death rate from alcohol induced liver disease accounts for 40% of deaths from cirrhosis or 28% of all deaths from liver disease[2]. Alcoholic liver disease is one of the top ten leading causes of death in developed countries, responsible for 3% (1.8 million) of all deaths[3]. While alcoholic hepatitis is common, its pathogenesis, predictors for survival, and treatment remain debated.

There have been several excellent reviews on the treatment of alcoholic hepatitis in the past year[4,5]. The focus of our review is to expand on the treatment of alcoholic hepatitis while addressing the role of scoring systems and liver biopsy.

PATHOGENESIS

While the histology of alcoholic hepatitis is well characterized, the pathogenesis remains uncertain. A number of hepatocellular and inflammatory processes including the potential involvement of innate and adaptive immunologic responses are under investigation.

The variety of treatment options in alcoholic hepatitis share a common treatment goal of blocking the myriad of innate immunologic responses which include macrophage release of chemokines and cytokines, TNF-α, IL-1β, IL-6 and IL-8, in addition to adaptive immune responses to acetaldehyde and hydroxyethyl radical formation[6,7].

While the immunologic responses are varied, they share similar end results of apoptosis, necrosis, inflammation and fibrosis. The history and basis of treatment interventions in alcoholic hepatitis are centered on blocking one or more of the harmful mechanisms found in the animal model with the hope, in human trials, of providing survival benefit (Table 1).

SCORING SYSTEMS

There are several scoring systems applied toward predicting survival in alcoholic hepatitis. Many clinically employed scoring systems, however, are derived from related liver diseases and later validated for alcoholic hepatitis. As patient risk stratification and allocation of treatment are entirely dependent on a system to gauge short-term mortality, it is important to develop a scoring system that accurately predicts survival versus mortality.

First developed as risk stratification method for patients undergoing shunt surgery and later refined[8,9], the Child Turcotte Pugh (CTP), score has evolved as the

Table 1 Immunologic responses and directed therapies in alcoholic hepatitis

Cytokines	Mechanism	Mediators	Treatment
Cytokines			
TNFα	Via TNFR1 signaling in hepatocytes and Kupffer cells (KC)	Apoptosis, necrosis, KC production of cytokines, potential cytoprotective effect	Glucocorticoids; Pentoxifylline; Infliximab
IL-6, 8	Lymphocyte and neutrophil activation, release of acute phase reactants	Inflammation, fibrosis	Glucocorticoids
Antigenic adduct	Oxidation of ethanol, binding to proteins forming antigenic adducts	Adaptive immunity	Glucocorticoids
Chemokines	Attract leukocytes and increase adhesion molecules	Inflammation	Glucocorticoids
Oxidative Injury			
S-adenosyl-methionine (SAMe)	Precursor for glutathione, defense mechanism against oxidative stress, increase methylation	Protective role of SAMe	SAMe
Hypoxia/ischemic injury	Hypermetabolic state	Insufficient oxygen	Propylthiouracil

most clinically used prognostic tool in cirrhotic patients. Patients are stratified into three categories based on point assignment of objective and subjective measures of liver function (Appendix A). The clinical application of the CTP score applied to cirrhotic patients has been validated[10,11]. Cited over 1700 times, the CTP score remains one of the most important clinical predicting tools in patients with cirrhosis[12]. However, in the setting of alcoholic hepatitis, there is evidence that other scoring systems may better predict survival.

The Maddrey Discriminant Function (DF) score, unlike the CTP, is derived from a clinical trial studying the efficacy of corticosteroid therapy in patients with alcoholic hepatitis[13]. Admission prothrombin time and serum bilirubin are independently and significantly associated with mortality and serve as the key variables in calculating the DF score. Further modified, in combination with the presence of encephalopathy, a DF score greater than 32 predicts greater than 50% mortality[14]. The DF score has subsequently been used to stratify patients in trials studying the efficacy of corticosteroid treatment in patients with alcoholic hepatitis[14,15]. While the DF score provides risk stratification, concerns of center to center variability of prothrombin time measurement[16], significant mortality in patients with DF score less than 32 and low test specificity have led some investigators to suggest alternative scoring systems[17].

The Mayo Endstage Liver Disease (MELD) Score, like the CTP score, was developed to predict survival in patients undergoing decompressive therapy for portal hypertension[18,19] (Appendix A). Unlike the CTP score, the MELD score is derived from prospective data and lacks subjective measurements of liver function. Using a derived formula, the MELD score is calculated using prothrombin time, creatinine and bilirubin. All of which are factors Maddrey *et al* found to be significant in his original study. However, unlike the Maddrey Discriminant Function, the MELD score is employed as a continuous assessment of liver function and includes creatinine, a marker of development of hepatorenal syndrome. It would be interesting to evaluate the Maddrey DF as a continuous marker of liver function.

The MELD score has been validated in predicting survival in patients with end-stage liver disease and chronic liver disease[19,20]. Three retrospective studies suggest that MELD is equivalent to DF in predicting survival in patients with alcoholic hepatitis[22,23]. One study found MELD to be more predictive of survival if calculated after the first week of hospitalization[23]. This result may reflect the fact that patients with a higher likelihood of dying decline in the first few weeks of hospitalization rather than improved test sensitivity. In summary, admission MELD, DF and CPT did not differ significantly from each other (Table 2).

Citing concerns of low specificity of the Maddrey DF score and the difficulty of identifying an optimal cut-off point of the MELD score[24,25], Forrest *et al* introduced the Glasgow alcoholic hepatitis score (GAHS)[16] (Appendix A). Using retrospective data from patients presenting with alcoholic liver disease, the authors use stepwise logistic regression to identify variables associated with mortality. In Table 2, we include the results of Forrest *et al*.'s study with separate comparisons between GAHS *versus* DF and GAHS *versus* MELD from the validation portion of the trial. We compared the accuracies of each test *via* Chi-square analysis. GAHS is more accurate than DF in 28 and 84 d mortality prediction but equivalent to MELD in 28 d mortality prediction. The reported specificities of both MELD and DF in this study are quite low, especially in comparison with other studies. However, the reported low specificity of DF significantly affected its comparative predictive capacity, *via* chi-square analysis, against GAHS.

Alcoholic hepatitis scoring systems are in evolution. MELD and DF appear to be equivalent in predicting mortality. The higher specificity and overall accuracy of the GAHS, if confirmed, may make it a better screening tool in the clinical trial. It would be interesting to review previous studies assessing the efficacy of glucocorticoid treatment using GAHS as the scoring system. For clinicians, however, a test with a higher sensitivity would be desirable as their primary goal is to maximize the number of patients receiving a treatment benefit. Therefore, the DF score may be preferred in the clinical setting as

Table 2 Sensitivity and specificity of scoring systems for alcoholic hepatitis: sensitivities (Sen)/specificities (Spec)

Study	Patients	Study design	Predictive Mortality	MELD Sen/Spec (%)	DF Sen/Spec (%)	CTP Sen/Spec (%)	Glasgow Sen/Spec (%)	Conclusions
Sheth *et al*[21] 2002	34	Retrospective	30 d	⩾ 11 86/82	⩾ 32 86/48	N/A	N/A	MELD equivalent to DF
Kulkarni *et al*[17] 2004	41	Retrospective	28 d	N/A	⩾ 33 66.7/61.5	N/A	N/A	DF ⩾ 32 is appropriate. High mortality in DF < 32
Dunn *et al*[22] 2005	73	Retrospective	90 d	⩾ 21 75/75	⩾ 37 88/65	N/A	N/A	MELD equivalent to DF
Srikureja *et al*[23] 2005	202	Retrospective	Not given	Admission: ⩾ 18 85/84 Wk 1: ⩾ 20 91/85	⩾ 32 83/84	⩾ 12 76/80	N/A	Admission MELD equivalent to DF
Forrest *et al*[16] 2005	134	Retrospective	28 d 84 d	N/A	⩾ 32 28 d 96/27 84 d 95/31	N/A	⩾ 9 28 d 81/61 84 d 78/66	GAHS more accurate in predicting mortality compared to DF
Forrest *et al*[16] 2005	46	Retrospective	28 d 84 d	⩾ 11 28 d 92/29 84 d 92/29	N/A	N/A	⩾ 9 28 d 75/68 84 d 69/67	GAHS more accurate in predicting 84 d mortality GAHS equivalent to MELD in predicting 28 d mortality

it captures more patients at risk of dying than does the GAHS score.

LIVER BIOPSY

Since it was reported first by Mallory *et al*[26] the morphology of alcoholic liver disease has been well described[27]. The details and significance of morphologic progression from steatosis to cirrhosis, as it relates to survival and treatment, continue to be refined.

Employed as part of an entry and stratification criteria in a few clinical trials[13,15,28-31], liver biopsy for staging and predicting survival has been replaced by scoring systems. Although survival did not differ between the glucocorticoid trials that used pre-randomization biopsies versus scoring system, we think that clinical trials should enroll biopsy-proven cases. Biopsy confirmation of alcoholic hepatitis accurately defines the patients eligible for clinical trials and, in our view, is preferred if fever, leukocytosis and hepatic bruit are absent.

While histologic changes from steatosis and steatohepatitis to cirrhosis are known, correlating degree of steatosis with liver function and survival is currently under investigation. A study by Duvoux *et al*[32] finds a correlation between low grade steatosis and advanced liver failure as well as lowered sensitivity to steroid treatment. However, patients with low grade steatosis had higher Maddrey discriminant function scores, which can also predict poor survivals.

The role of liver biopsy in defining prognosis and treatment of alcoholic hepatitis in the clinical setting remains unclear. A thorough patient history and physical examination has a reported sensitivity and specificity of 91% and 96% in diagnosing alcoholic hepatitis[33]. However, from the above study, four cases out of 103 were misdiagnosed as alcoholic hepatitis upon review of biopsy specimen. It is generally accepted to perform a liver biopsy if the diagnosis of alcoholic hepatitis is either in question or a concomitant pathology, such as hepatitis C, is suspected[34]. Approximately 35%-40% of alcoholics are infected with HCV[35,36] and experience higher mortality rates than patients with alcoholic liver disease alone[37,38].

A patient history and physical exam cannot, however, consistently distinguish between and determine the extent of alcoholic hepatitis alone versus alcoholic hepatitis with concomitant cirrhosis. A biopsy can provide useful prognostic and diagnostic information. Patients with alcoholic hepatitis and cirrhosis have significantly higher 1- and 5-year mortality compared to patients with cirrhosis alone[39,40]. Presence of perivenular fibrosis, steatosis and giant mitochondria in a known alcoholic may herald the transition from alcoholic hepatitis to cirrhosis; a transition which could be prevented with abstinence[41-43]. In clinical practice, we recommend transjugular(given the presence of coagulopathy and/or ascites) liver biopsy in cases in which it is difficult to distinguish the contribution of alcoholic hepatitis and end-stage cirrhosis, especially when the hallmarks of alcoholic hepatitis, leukocytosis, fever and hepatic bruit are absent. Since treatments are associated with complications, we believe it is prudent to be confident of the diagnosis before using steroids.

TREATMENT

Glucocorticoids

First studied in the treatment of alcoholic cirrhosis in

Table 3 Randomized controlled glucocorticoid trials in treatment of alcoholic hepatitis (% Death)

Study	Glucocorticoid	Patient	Severity assessment	HE	Placebo All (%) / HE (%)	Steroid All (%) / HE (%)	RRR (95% CI) All % / HE %	NNT (95% CI) All / HE	Quality score
Porter *et al*[125] 1971	Methylprednisolone	20	Self derived	16	7/9 (77)[b] 7/8 (88)[c]	6/11 (55)[b] 6/8 (75)[c]	N/A	N/A	5
Helman *et al*[28][a] 1971	Prednisolone	37	Self derived	15	6/17 (35) 6/6 (100)	1/20 (5) 1/9 (11)	86 (-0.06-0.98) 84 (0.28-0.96)	3 (2-18) 1 (1-2)	2
Campra *et al*[31] 1973	Prednisone	54	Self derived	18	9/25 (36) 8/10 (80)	7/20 (35) 4/8 (50)	N/A	N/A	2
Blitzer *et al*[52] 1977	Prednisolone	28	Self derived	5	5/16 (31) 1/2 (50)	6/12 (50) 2/3 (67)	N/A	N/A	5
Lesesne *et al*[49][a] 1978	Prednisolone	14	Self derived	14	7/7 (100) 7/7 (100)	2/7 (29) 2/7 (29)	67 (0.05-0.88) 67 (0.05-0.88)	2 (1-4) 2 (1-4)	3
Shumaker *et al*[30] 1978	Methylprednisolone	27	Self derived	12	7/15 (47) 4/6 (67)	6/12 (50) 2/6 (33)	N/A	N/A	5
Maddrey *et al*[13][a] 1978	Prednisolone	55	DF	15	6/31 (19) 6/10 (60)	1/24 (4) 1/5 (20)	79 (-0.67-0.97) 67 (-1.1-0.95)	6 (-3-111) 3 (-1-16)	4
Depew *et al*[126] 1980	Prednisone	28	Self derived	28	7/13 (54) 7/13 (54)	8/15 (53) 8/15 (53)	N/A	N/A	4
Theodossi *et al*[53] 1982	Methylprednisolone	55	Self derived	34	16/28 (57) 10/14 (71)	17/27 (63) 17/20 (85)	N/A	N/A	3
Mendenhall *et al*[48] 1984	Prednisolone	178	Self derived	61	50/88 (57) 10/30 (33)	55/90 (61) 11/31 (36)	N/A	N/A	3
Carithers *et al*[14][a] 1989	Methylprednisolone	66	DF	33	11/31 (36) 9/19 (47)	2/35 (6) 1/14 (7)	84 (0.3-0.96) 85 (-0.06-0.98)	3 (2-9) 2 (2-7)	5
Ramond *et al*[15][a] 1992	Prednisolone	61	DF	19	16/29 (55) 7/10 (70)	4/32 (13) 2/9 (22)	77 (0.4-0.9) 68 (-0.15-0.9)	2 (2-5) 2 (1-12)	5

[a]$P < 0.05$ for survival benefit as reported by study authors; Note percent death at 28 d in placebo *versus* steroids is shown in each box for all cases[b] and for those with hepatic encephalopathy. [c]RRR, NNT is calculated from published data of those studies that reported a significant survival benefit. HE: Hepatic Encephalopathy; DF: Maddrey Discriminant Factor; CP: Child Turcotte Pugh; N/A: non significant difference in mortality as reported by study authors; Self derived: Criteria derived by study team not including CP, DF or MELD.

1960[44], the use of glucocorticoids remains perhaps the most studied and debated intervention. Reported successes of glucocorticoids are variable and appear largely dependent on the nature of the trial.

The rationale for the use of glucocorticoids is centered upon blocking the cytotoxic and inflammatory pathways in alcoholic hepatitis. Glucocorticoids decrease circulating inflammatory cytokines such as TNF-α, ICAM-1 expression, and have demonstrated short term histologic improvement in the treatment of alcoholic hepatitis[45].

It is difficult to provide a simple summary of results for glucocorticoid trials in alcoholic hepatitis. While all trials appeared to have been controlled, few have high quality scores given the variable definition of randomization and blinding in each trial[46,47].

The trials vary by inclusion/exclusion criteria, glucocorticoid type, scoring system, length of treatment and co-interventions. The study by Mendenhall *et al* is particularly difficult to interpret as essentially three different intervention arms, oxandrolone, prednisolone and prednisone, are employed[48]. Lesesne *et al* compares prednisolone to a 1600 caloric intake diet which is below the estimated caloric needs of most hospitalized patients; his trial is not placebo controlled[49]. Furthermore, the variation in type of glucorticoid, dose, and treatment length makes it difficult to provide treatment guidelines for physicians.

We did a computerized search using the MEDLINE database from 1971 to August 2005 using the search headings of "steroids", "corticosteroids", "alcoholic hepatitis", "hepatitis, alcohol", "randomized" and "English". We obtained additional trials by manually searching through retrieved trials and review articles. Randomized trials including corticosteroids in the treatment of alcoholic hepatitis with the outcome measure of mortality are summarized in Table 3. The trial results are summarized by percentage death in each group including number of patients with hepatic encephalopathy and their percentage mortality. Relative risk reduction (RRR), number needed to treat (NNT) with their associated 95% confidence intervals are calculated for trials that reported a significant benefit in survival. Given that hepatic encephalopathy is a known predictor of mortality, we have also calculated RRR and NNT for those patients; again only in trials that reported a significant survival benefit. We employed the Jadad score as an assessment of trial quality. The Jadad score is one of the few validated measures of randomized trial quality[46,50,51]. Out of a maximum score of five, points are assigned based on the method of randomization, double blinding and description of withdrawals/drop-outs.

The results are variable. Five trials reported a significant

survival benefit with glucocorticoids[13-15,28,49]. RRR ranged from 67% to 86%, with NNT varying between 2 and 6. As reported in the above trials and noted in our table, glucocorticoid treatment significantly reduces mortality in patients with hepatic encephalopathy. The latter trials by Carithers *et al* and Ramond *et al*, selectively treated patients with discriminant function scores greater than 32, supporting a more discriminate use of glucocorticoids[14,15].

Of the trials which reported a non-significant benefit, there are four trials which report higher mortality in the glucocorticoid group[30,48,52,53]. While glucocorticoids are relatively benign in the short term for most patients, the remaining three trials remind clinicians that there are significant complications with their use. Blitzer *et al* reported a higher number of fungal infections in the steroid group contributing to the greater percentage of deaths in the steroid group when compared to placebo. However, his steroid treatment group contains a higher proportion of patients with elevated total bilirubin, when compared to placebo, which may contribute to his study result[52]. Included in our discussion of nutrition in the treatment of alcoholic hepatitis, Cabre *et al*'s study found 31% (11/35) mortality in the steroid group with 91% of deaths attributable to infection[54]. It is important to recognize the potentially serious infectious complications secondary to steroid treatment.

Subsequent meta-analyses, while still yielding conflicting results, began to delineate which patients would most benefit from glucorticoid treatment. The first meta-analysis on this topic, conducted by Imperiale and McCullough in 1990 (which antedates more recent trials), finds a protective efficacy of glucocorticoids in higher quality trials, particularly those that exclude patients with gastrointestinal bleeding but include patients with hepatic encephalopathy[55]. They found a protective efficacy of 34% overall (95% CI, 15%-48%) for patients with hepatic encephalopathy. Imperiale and McCullough's study applies quality scores which are important in the setting of such trial heterogeneity. In their paper, quality scores are assigned by independent assessors. Quality scores we use in this paper are derived from Jadad *et al*, and have been used in assessing the quality of randomized clinical trials[46]. We do not find a significant association between high Jadad score and trial survival benefit. However, Jadad *et al* score does not consider baseline equivalence of compared groups, use of co-therapies and adequate potency of principal therapy. The variance of survival amongst the trials may have more to do with patient inclusion/exclusion criteria and the self-derived scoring systems than trial quality and adherence to randomization and double blinding.

Meta-analysis conducted by Christensen *et al*, did not find an overall treatment benefit, after attempting to control for confounding variables[56]. Controlling for confounding variables without direct access to individual study data can be difficult given the heterogeneity of prior trials. A subsequent study, by Mathurin, Mendenhall, Carithers *et al*[57] pooled raw data from their respective trials based on DF score (greater than 200 patients with DF $\geqslant$ 32 in placebo versus steroids) and found a survival benefit. If the DF < 32, there was a > 90% survival without steroids. The conclusions from the above study provide a more definitive treatment guideline for clinicians. In patients with DF $\geqslant$ 32, treatment with glucocorticoids improves short term, 28 d, survival with mortality decreasing from 35% in controls to 15% with steroids.

The longer term benefit of glucocorticoids are difficult to assess given the variable long- term clinical trial definitions (1.5 mo, 6 mo, 1 year)[54,48,58] and each of the existing three trials reported different outcomes: harm, no benefit and benefit[54,48,58]. It is also difficult to assess long-term benefit as alcoholic hepatitis is likely to recur unless the patient abstains.

Recommendations

A review of the literature supports a more discriminate use of glucocorticoids in patients with a Maddrey discriminant function score $\geqslant$ 32. If there is no evidence of gastrointestinal bleeding or infection, the frequent concomitant presence of hepatic encephalopathy provides an even stronger support for the use of glucocorticoids. A study by Mathurin *et al* suggests a simple method to identify patients who are most likely to respond to glucocorticoids. Patients with an 'early change in bilirubin levels' (ECBL), i.e. a bilirubin level at 7 d lower than the bilirubin level on the first day of treatment, were significantly more likely to survive and respond to steroid treatment[59]. Discontinuation of glucocorticoid treatment in the non-responder group, i.e. patients that did not have an ECBL at 7 d, did not appear to result in adverse events[5]. Devising methods to target the patient groups most likely to benefit are important in maximizing treatment benefit, avoiding unnecessary complications of treatment and streamlining the treatment decision process.

Glucocorticoids, while providing a benefit in a select group of patients, are not without risks and should be used with caution in patients with infectious complications and gastrointestinal bleeding. Further trials that are larger in sample size, involving multiple centers and with an active comparator, i.e. pentoxifylline, are needed to better delineate the true effect of glucocorticoids. Finally, as scoring systems are changing, repeat studies may be needed to reassess the treatment effect of glucocorticoids employing MELD and GAHS.

Anabolic steroids

There is a measurable and clinically apparent decline in gonadal function in patients with alcoholic liver disease[60-62]. In 1938, administration of androgens appeared to enlarge the liver of cirrhotic rats, thereby suggesting that perhaps androgens could reverse the process of fibrosis[63]. This lead to the first clinical trial where 12 patients with alcoholic cirrhosis were injected with large doses of testosterone with 'some improvement'[64].

A 2003 Cochrane systematic review could not demonstrate a significant effect of anabolic-androgenic steroids on the mortality of patients with alcoholic liver disease[65]. Three trials[48,66,67] included in the analysis and two trials[68,69] excluded from the analysis are trials in which all participants have alcoholic hepatitis.

Table 4 Infliximab trials in the treatment of alcoholic hepatitis

Study	Design	Patients	Treatment	Results
Spahr *et al*[76] 2002	Randomized	20	All patients: prednisone for 28 d Randomized d 0 R1: Infliximab 5 mg/kg R2: Placebo	Improved Maddrey score No significant difference in survival, histology or adverse outcomes
Tilg *et al*[77] 2003	Case Series	12	Infliximab 5 mg/kg	83% (10/12) survived at median 15 mo No mention of infection
Mookerjee *et al*[78] 2003	Case Series	10	Infliximab 5 mg/kg times one	72 h assessment Significant reduction in laboratory parameters Increased hepatic and renal blood flow
Naveau *et al*[79] 2004	Randomized	36	All patients: Prednisone for 28 d R1: Infliximab 10 mg/kg R2: Placebo	Significantly higher rate of infections in treated group Non-significantly higher rate of death in treated group Study stopped secondary to adverse events in treatment group

Reflective of the conclusions derived from the systematic review, Bonkovsky *et al*'s study[67] and Mendenhall *et al*'s 1984 study[48] did not find a significant survival advantage in the anabolic steroid group in the placebo. In Mendenhall *et al*'s study, patients in the oxandrolone group were treated for 30 d. They report, however, in subgroup analysis that patients with moderate hepatitis treated with oxandrolone seemed to have survival advantage 6 mo post treatment. As the subgroup analysis did not include patients that had died within the first two months of treatment, the results should be taken with some caution.

Currently, anabolic steroids are not recommended for the treatment of alcoholic hepatitis.

Pentoxifylline

Pentoxifylline is a suppressor of tumor necrosis factor alpha (TNF-α), prevents leukocyte adherence to vascular endothelium and down regulates the expression of intercellular adhesion molecule-1 in monocytes[70]. The main signaling pathway is through type 1 tumor necrosis factor receptor, TNFR1. Elevated levels of TNF-α are predictive of poor survival in alcoholic hepatitis[71,72]. Other effects of this drug may contribute to its action such as its effects on membrane fluidity which determine its use in peripheral vascular disease.

First studied by McHutchison *et al* in 1991, in patients with severe alcoholic hepatitis (defined as DF score $\geqslant$ 32), pentoxifylline reduced the development of hepatorenal syndrome, and as a consequence mortality, in comparison to patients who received placebo[73]. A subsequent double blind placebo controlled trial, Akriviadis *et al*, from the same center, supports McHutchison's findings[74]. There did not appear to be any complications as a consequence of pentoxifylline treatment. As noted by Dr. Mathurin[5], the latter study showed no improvement in liver function tests. The reported improved survival was accounted for by a reduction in the development of hepatorenal syndrome in the treatment group. This finding is in sharp contrast to the glucocorticoid trials which demonstrate an improvement in liver function and in survival compared to placebo.

A small sample size, retrospective, observational study by McAvoy *et al*, published as an abstract, finds a treatment benefit with pentoxifylline only in patients stratified to GAHS $\geqslant$ 9, but not in patients with DF $\geqslant$ 32[75]. As raw numbers are not available at press time, it is difficult to draw a meaningful conclusion from his study.

Recommendations: Pentoxifylline may reduce mortality from hepatorenal syndrome in the setting of severe alcoholic hepatitis but further studies are needed to confirm these findings. Aside from the need for head to head comparative trials with steroids, one wonders if the combination of the two treatments might exhibit an additive benefit.

Infliximab

Infliximab, used in the treatment of Crohn's disease, rheumatoid arthritis and psoriasis, is a chimeric mouse/human antibody which binds to tumor necrosis alpha, blocking its effects[76]. Preliminary trial data was encouraging. Three trials reported either better survival than predicted, improved Maddrey score or laboratory parameters[76-78]. The largest and most comprehensive trial studying the efficacy of prednisone and infliximab in the treatment of alcoholic hepatitis was terminated early when a significantly higher number of deaths occurred in the treatment group[79]. The study received some criticism for its use of high dose of infliximab and infusion protocol which varied from previous studies. In this study, investigators cited prior studies in Crohn's[80] and rheumatoid arthritis[81] in which there did not appear to be a relationship between dose of infliximab and rate of infection[78]. Furthermore, Dr. Naveau contends, perhaps infliximab is not the TNF-α blocking agent for alcoholic hepatitis (Table 4).

An open label uncontrolled pilot study on etanercept in the treatment of moderate to severe alcoholic hepatitis was completed[82]. Of the 13 patients treated, 7 had Maddrey DF greater than 32 and two of the seven died within 32 d. Etanercept was discontinued in 3 patients secondary to infection, hepatorenal decompensation and gastrointestinal bleeding. Therefore, there is no particular evidence one way or the other to suggest a beneficial or detrimental effect of treatment. As etanercept targets soluble TNF, whereas infliximab targets both soluble and

membrane bound TNF, it is uncertain what the advantages/disadvantages are of this distinction in the setting of this specific disease target.

Infliximab is not currently recommended for the treatment of alcoholic hepatitis, outside of clinical trials. Although concerns have been raised about increased risk of infection, the more disturbing aspect has been recent warnings of acute liver failure in patients with Crohn's disease and rheumatoid arthritis treated with infliximab[83]. This risk may preclude its use in patients with underlying severe liver injury who are less capable of withstanding an additional insult to the liver.

Nutrition

There are multiple etiologies for weight loss and malnutrition in patients with years of alcohol abuse. Weight loss can be reflective of years of substitution of alcohol for more than 50% of other calories[84], malabsorption of dietary fat and nutrients[85] and the induction of a catabolic state resulting in skeletal muscle depletion[86].

Recently reviewed[4] parenteral and enteral nutrition, while improving liver function in a few studies in alcoholic hepatitis[87-89] has yet to demonstrate a change in clinical outcome.

Mendenhall *et al* have done the most extensive assessment on the effect of protein calorie malnutrition (PCM) and protein energy malnutrition (PEM) on survival and liver function. The results of their interventional studies are shown in Table 5. The observed associations between degree of malnutrition, as calculated by PCM or PEM score, and severity of liver disease[90-92] as well as improvement of survival with improved PCM score[93] serve as the basis for determining the effect of nutritional intervention on survival and liver function in alcoholic hepatitis[69,94].

Nutritional interventions such as caloric amount, type, mode and duration of supplementation vary among the trials. For example, the 1600 caloric nutritional intervention in Lesesne *et al*'s study is below that of most hospitalized patients. A positive correlation between nutritional intake and survival, if present, would not be expected. Much as in the case of the glucocorticoid literature, it would be difficult to provide clinical recommendations when the treatment interventions and outcomes vary. Furthermore, it is difficult to draw meaningful clinical conclusions. While nitrogen balance improves in the nutrition intervention arm, survival remains unchanged.

The majority of trials did not find a survival advantage in nutritional support. There are two trials which showed a survival advantage. Nasrallah *et al*'s study is smaller and both groups receive a 3000 kCal diet with protein, which is an intervention treatment in some studies. Mendenhall *et al,* found a later survival advantage, 6 mo post treatment, in the moderately malnourished group. This is in contrast to Mezey *et al*'s study which did not find a survival advantage up to two years after treatment.

Recommendations: It is important to assess nutritional status of patients in order to recognize and treat the distinct nutritional deficiencies inherent in alcoholic cirrhosis and hepatitis. At this time, however, nutritional supplementation during acute presentation of alcoholic hepatitis does not appear to affect survival.

Colchicine

The final histologic stage in alcoholic liver disease is cirrhosis. Found to inhibit liver fibrosis in rats[95], colchicine's anti-fibrotic activity presented a theoretical possibility of preventing liver fibrosis in humans.

Three clinical trials in the setting of alcoholic hepatitis[96,97] and a Cochrane database review in the setting of alcoholic and non-alcoholic liver fibrosis[98] fail to find a benefit in the treatment of alcoholic hepatitis with colchicine. Recently published and not included in the cochrane review, the largest trial studying long-term colchicine in the setting of alcoholic cirrhosis did not find a therapeutic benefit when compared to placebo, in concordance with prior literature[99]. Colchicine is not currently recommended for the treatment of alcoholic hepatitis.

S-adenosyl-methionine

SAMe, produced from methionine by adenosylmethionine synthetase, is important in the metabolism of nucleic acids, structure and function of cell membranes and as a precursor of glutathione. Glutathione may be protective in alcohol induced liver injury[100]. However, in liver disease there is an impairment of enzyme activation of methionine which cannot be corrected by methionine supplementation[101]. In the setting of alcoholic hepatitis, there is a measurable decrease in hepatic methionine, SAMe and glutathione levels[102]. In animal studies, administration of SAMe increased glutathione levels, attenuated ethanol induced liver injury as well as liver injury caused by other hepatotoxins[103-106].

In a 2001 Cochrane systematic review[107], SAMe has yet to consistently demonstrate a significant beneficial effect on the mortality in the setting of alcoholic liver disease. None of the analyzed trials in the systematic review targeted patients with alcoholic hepatitis. The largest multi-center and highest Jadad quality scoring trial, by Mato *et al*, treated patients with alcoholic cirrhosis with SAMe for up to two years[108]. There was an overall decline in mortality in the treatment group compared to placebo, but did not reach significance. Excluding patients with Child's C cirrhosis, however, did yield a significant mortality benefit.

There are currently two NIH funded trials studying the effect of SAMe on the mortality in the setting of alcoholic cirrhosis. There has yet to be a trial studying the effect of SAMe administration on survival in the setting of acute alcoholic hepatitis.

SAMe is currently not recommended in the treatment of acute alcoholic hepatitis.

Propylthiouracil (PTU)

Found to reduce hypoxic hepatocellular injury in ethanol fed rats[109], subsequent animal studies confirm PTU's protective role against oxidative and ischemic liver injury[110]; similar hepatic injuries are found in patients with alcoholic hepatitis[111].

In a 2001 Cochrane systematic review[112], PTU did

Table 5 Interventional studies on nutrition and alcoholic hepatitis

Study	Design	Patients	Intervention	Findings
Lesesne *et al*[49] 1978	Randomized	14 patients, alcoholic hepatitis and encephalopathy	7 controls, 1600 Kcal diet 7 study, prednisolone	Reduction in mortality in the prednisolone arm
Galambos *et al*[127] 1979	Case series	11 patients, alcoholic hepatitis	4, enteral hyperalimentation 7, parenteral hyperalimentation	No difference in mortality Increased nitrogen balance in study group
Nasrallah *et al*[128] 1980	Randomized	35 patients, alcoholic hepatitis	All received 3000 kcal 100g protein diet 18 control 17 study, 70-85 gram of intravenous amino acid	Lower mortality in the study group
Diehl *et al*[129] 1985	Randomized	15 patients, alcoholic hepatitis	All allowed to consume hospital diet ad libitum 10 controls, glucose solution 5 study, glucose solution + amino acids	Increased nitrogen balance in study group No difference in clinical and biochemical markers of liver disease
Mendenhall *et al*[94] 1985	Randomized	57 patients, moderate-severe alcoholic hepatitis	34 controls, 2500 cal diet 23 study, Hospital diet + Hepatic Aid	No difference in mortality Improvement in nutritional parameters in intervention group
Calvey *et al*[130] 1985	Randomized	64 patients, alcoholic hepatitis	32 controls, standard diet 32 study, standard diet + 2000 kCal + 10 g nitrogen	No difference in biochemical or clinical parameters
Soberon *et al*[131] 1987	Case series	14 patients, alcoholic hepatitis	6 with adequate nutritional status, hospital diet 8 with poor baseline nutritional status, nasoduodenal diet, 35 kCal/kg per day	No difference in mortality Increased nitrogen balance in study group
Simon *et al*[87] 1988	Randomized	12 patients, moderate alcoholic hepatitis 22 patients, severe alcoholic hepatitis	Moderate Group 6 control, standard diet 6 study, PPN Severe Group 12 control, standard 10 study, PPN	No difference in mortality Improved in biochemical tests in severe group
Bonkovsky *et al*[67] 1991	Randomized	39 patients, moderate to severe alcoholic hepatitis	9, standard therapy 8, oxandrolone + standard therapy 10, PPN 12, oxandrolone + standard therapy + PPN	Improved biochemical parameters
Mezey *et al*[88] 1991	Randomized	52 patients, alcoholic hepatitis	28 control, dextrose solution 26 study, dextrose + amino acid	No difference in mortality during hospitalization and 2 yr after treatment
Mendenhall *et al*[69] 1993	Randomized	273 patients, severe alcoholic hepatitis	136 control 137 study, oxadrolone + enteral nutrition	No difference in mortality overall Improvement in mortality in moderately malnourished group(19%) versus control (51%) at 6 mo post treatment
Cabre *et al*[54] 2000	Randomized	71 patients, severe alcoholic hepatitis	36, prednisolone 35, enteral tube 2000 kCal/d	No difference in overall mortality Higher early mortality in nutrition *versus* higher follow up mortality on steroids
Alvarez *et al*[132] 2004	Case series	13 patients, severe alcoholic hepatitis	13, prednisolone + TEN 2000 kCal/d	15% death during treatment 67% of patients developed infections during treatment -no deaths due to infections

not provide a significant survival benefit in the setting of alcoholic liver disease. All of the analyzed six studies (3 of which were published only in abstract) included patients with alcoholic hepatitis[110,113-117].

Contrary to animal studies, hepatic histologic improvement with PTU administration is not replicated in clinical trial literature. PTU also does not appear to have a measurable effect on splanchnic hemodynamics in the setting of alcoholic cirrhosis[118].

While the systematic review did not find a significant association between PTU and adverse events, one trial was discontinued when higher mortality rates were observed in the PTU group[110]. Furthermore, there are case reports and several reviews on fulminant hepatic failure and hepatitis[119-123] secondary to PTU in addition to leukopenia[124] Propylthiouracil is not recommended for the treatment of alcoholic hepatitis.

CONCLUSIONS

The treatment of alcoholic hepatitis continues to evolve as our understanding of the disease process expands. As it does so, however, it is important that our clinical trials attempt to achieve the highest quality possible. Trials

designed to replicate treatment effect should be done with treatment dosages and duration that can be employed in the clinical setting.

Further modification of scoring systems and streamlining methods to identify patients most likely to respond to treatment continue to improve as we seek to minimize risk of treatment while maximizing survival gain.

At the present, we recommend corticosteroids for patients with alcoholic hepatitis and DF $\geqslant$ 32, providing there is not evidence of gastrointestinal bleeding. In patients with active infection, we delay treatment until antibiotic control of infection is achieved. Given the various glucocorticoids and dosages employed in clinical trials, it is difficult to provide clinicians evidence based guidelines on type of glucocorticoid, dosage and length of treatment. We currently recommend using the lowest effective dose of prednisone or prednisolone studied in the literature. As prednisone is less costly, we prescribe prednisone 40 mg daily for up to 28 d. If no improvement in bilirubin is seen after 7 d, we recommend stopping glucocorticoids as suggested by Mathurin[59]; switching to pentoxifylline is a reasonable alternative in that situation. Although primary treatment with pentoxifylline holds some promise, the evidence of its efficacy is not as robust as that with steroids.

Although the focus of this article is treatment, preventing the occurrence of disease is important. From physician screening for alcohol abuse to community wide education in a culturally sensitive manner on the risks of alcohol abuse are important health service fields.

APPENDIX A

Child-Turcotte with pugh modification

Score	1	2	3
Prothrombin time (INR)	< 4 s (< 1.7)	4-6 s (1.7-2.3)	> 6 s (> 2.3)
Bilirubin (mg/dL)	< 2	2-3	> 3
Albumin (g/dL)	> 3.5	3.5-2.8	< 2.8
Ascites	None	Slight	Moderate
Encephalopathy	0	1-2	3-4

Maddrey criteria

	Score indicating
Initial	Poor prognosis
4.63 × prothrombin time (seconds) + serum bilirubin (mg/dL)	> 93
Modified	
4.6 (patients prothrombin time-control time) + serum bilirubin (mg/dL)	> 32

Glasgow alcoholic hepatitis score

Score	1	2	3
Age	< 50	$\geqslant$ 50	-
WCC (10^9 /L)	< 15	$\geqslant$ 15	-
Urea (mmol/L)	< 5	$\geqslant$ 5	-
PT ratio	< 1.5	1.5-2.0	> 2.0
Bilirubin (mmol/L)	< 125	125-250	> 250

GAHS $\geqslant$ 9 predictive of poor prognosis. MELD = 3.8 × $\log_e$ (bilirubin (mg/dL)) + 1.2 × $\log_e$ (INR) + 9.6 × $\log_e$ (creatinine (mg/dL)); One can also calculate the MELD score at the following internet address: www.mayoclinic.org/gi-rst/mayomodel7.html.

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EDITORIAL

Occult hepatitis C virus infection: A new form of hepatitis C

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Abstract

Occult hepatitis C virus (HCV) infection is a new recently characterized entity. This occult infection can be present in two different clinical situations: in anti-HCV negative, serum HCV-RNA negative patients with abnormal liver function tests and in anti-HCV positive subjects with normal values of liver enzymes and without serum HCV-RNA. This review describes recent studies of occult HCV infection in both kinds of patients.

Key words: Hepatitis C virus-RNA; Liver; Peripheral blood mononuclear cells

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INTRODUCTION

The etiology of liver disease is unknown in approximately 10% of patients with abnormal results on liver function tests. Some authors have reported that occult hepatitis B virus could be the cause of a proportion of these cryptogenic chronic hepatitis[1,2] cases, but no conclusive results have been yielded.

In January 2004, the role of occult hepatitis C virus (HCV) infection in chronic liver disease of unknown etiology was first described by Castillo *et al*[3]. This study included 100 patients with persistently long-standing abnormal liver function test results:alanine aminotransferase (ALT) and/or gamma-glutamyl transpeptidase (gamma-GTP). All known causes of liver diseases were excluded, and they were repeatedly anti-HCV and serum HCV-RNA negative. All these patients underwent a liver biopsy. A reverse-transcription polymerase chain reaction found that 57% of them had HCV-RNA in their liver. These results were also confirmed by *in situ* hybridization. In addition, 48/57 (84%) of these patients with occult HCV infection also had the antigenomic HCV-RNA strand in the liver tissue, indicating an ongoing viral replication. The HCV genotype found in the liver of the patients was 1b, which was demonstrated by a commercial genotyping assay and by amplification and sequencing of the HCV-core region. Moreover, 70% of the patients with intrahepatic HCV-RNA in liver also had viral RNA in their peripheral blood mononuclear cells (PBMC). Finally, liver necroinflammatory activity and fibrosis were observed in a significantly higher proportion of patients with occult HCV infection than in those without intrahepatic HCV-RNA. In summary, this paper identified a new form of hepatitis C virus infection called "occult HCV infection". It is characterized by the presence of HCV-RNA in the liver in the absence of serological markers of infection (anti-HCV and serum HCV-RNA negative).

The existence of this kind of occult HCV infection has also been found by other authors. Thus, Stapleton and colleagues have reported several studies on "seronegative" HCV infection in patients with cryptogenic liver disease and persistently abnormal results of liver tests[4,5]. Editorials or editors' comments have also been devoted to the role and significance of occult HCV infection, recognizing this infection as a new entity that should be taken into account for the diagnosis of patients with liver diseases of unknown cause[6-10].

Once occult HCV infection was identified, different research fields were developed: (1) To find an alternative to the liver biopsy for the diagnosis of occult HCV infection. (2) To study if HCV replicates or not in the PBMC of patients with occult HCV infection. (3) To compare the clinical, biochemical and histological characteristics of occult with chronic HCV infection. (4) To compare virus-specific T-cell responses in patients with occult and with chronic HCV infection. (5) To assess the possible role of occult HCV infection in the development of hepatocellular carcinoma. (6) To study the prevalence of occult HCV infection in other risk populations such as hemodialysis patients. (7) To assess the efficacy of antiviral therapy for occult HCV infection. (8) To study other possible clinical situations of occult HCV infection.

ALTERNATIVES IN DIAGNOSIS OF OCCULT HCV INFECTION

Although HCV-RNA is also detected in the PBMC of a high percentage of patients with an occult HCV infection, the gold standard for diagnosis of this occult viral infection

is detection of HCV-RNA in liver cells. However, because of the invasive nature of the liver biopsy, other alternatives were studied in an attempt to increase the sensitivity of the diagnostic tests in serum. Taking into account previous data recorded in patients with chronic hepatitis C[11], we performed a study with 21 patients diagnosed as having occult HCV infection (HCV-RNA positive in liver but negative in serum) and compared detection of viral RNA in plasma, PBMC and whole-blood[12]. All cases had negative results for HCV-RNA in plasma. In 3 (14%) patients, viral RNA was detected in whole-blood while HCV-RNA could be detected in PBMC of 57% of the included cases. Thus, using whole-blood as the source for HCV-RNA detection does not improve the sensitivity of the diagnosis of occult HCV infection. Testing for HCV-RNA in PBMC is much more reliable in identifing patients with an occult HCV infection when a liver biopsy is not available.

OCCULT HCV REPLICATION IN PBMC

One important question regarding the transmission of occult HCV infection was whether the virus could replicate in PBMC. To study this issue, 18 patients who had been diagnosed with occult hepatitis C by testing for HCV-RNA in their liver biopsy and who also had HCV in their PBMC were selected for this study[13]. By a strand-specific RT-PCR it was found that 61% of the patients had the antigenomic HCV-RNA strand in their PBMC, indicating that HCV was replicating in these cells. So, although the patients with occult HCV infection do not have detectable circulating virions, they could be potentially infectious.

CHRONIC VERSUS OCCULT HEPATITIS C

Once occult HCV infection is identified, one important question is if the clinical characteristics of this infection differ from those usually found in chronic hepatitis C. Trying to answer this question, the biochemical, virological and histological features of a group of 68 patients with occult HCV infection were compared with those of a group of 69 patients with histologically proven chronic hepatitis C[14]. Groups were matched with respect to gender, age and known time duration of the disease. Triglycerides and cholesterol values were significantly higher in occult HCV infection, while alanine aminotransferase, gammaglobulin, alpha-fetoprotein and iron levels were significantly higher in patients with chronic hepatitis C. The number of patients who had necroinflammatory activity and fibrosis in the liver biopsy was significantly higher in the group with chronic hepatitis C than in the group with occult HCV infection, but no difference was found in the percentage of patients with liver steatosis between both groups. Finally, as could be expected, the percentage of HCV-infected hepatocytes (determined by *in situ* hybridization) was significantly lower in patients with occult HCV. Thus, it was concluded that occult HCV infection is a milder disease, with less liver damage than chronic hepatitis C. Nevertheless, as patients with occult hepatitis C may present dyslipidemic disorders, studies on the natural history of occult HCV infection should be performed to prove the role of occult HCV as the cause for liver injury in these patients.

CELLULAR IMMUNE RESPONSES IN OCCULT HCV INFECTION

Why does occult HCV infection induce a less aggressive disease than chronic HCV infection? The immunological system of the patients could be involved in this situation.

Quiroga *et al*[15] have performed a study in an attempt to determine if the cellular immune response of patients with occult HCV infection is different from that of patients with chronic hepatitis C. This work compared 50 patients with occult HCV, 141 with chronic hepatitis C and 21 patients with cryptogenic liver disease (all known causes of liver disease were discarded, including an occult HCV infection). Overall, 26/50 (52%) of patients with occult HCV infection had $CD4^+$ T-cell proliferative responses. These responses were significantly more frequent in patients with occult HCV than in the group of patients with chronic hepatitis C (37/141: 26%; $P = 0.0016$) or in individuals with cryptogenic liver disease (1/21: 5%; $P < 0.001$). HCV-specific T-cells of patients with occult HCV infection proliferated more commonly in response to NS3 and NS4 proteins, and the peripheral blood mononuclear cells derived T-cell lines from these patients produced gamma interferon. Finally, patients with occult HCV infection had significantly higher amounts of HCV-specific $CD8^+$ T-cells than patients with chronic hepatitis C. In summary, HCV-specific cellular immune responses are more frequent in occult HCV infection than in chronic hepatitis C. Thus, patients with an occult HCV infection had a better immune response and this could be the cause of the milder disease that these patients have in comparison to those with chronic hepatitis.

These results seem to suggest that the clinical differences observed between occult and chronic hepatitis C are a consequence of the host's immunological system.

OCCULT HCV INFECTION AND HEPATOCELLULAR CARCINOMA

Hepatocellular carcinoma (HCC) is one of the most common malignancies throughout the world. In a small proportion of cases, the etiology agent associated with the development of liver cancer is unknown. Two different groups, one from Japan[16] and the other from Italy[17], have reported the presence of occult HCV infection in non-tumoral and tumoral liver tissues of patients with HCC who were negative to serological HCV markers. Therefore, it seems that occult HCV infection may play a role in the development of HCC however, as discussed above, more studies on this issue should be performed.

OCCULT HCV INFECTION IN RISK POPULATIONS

In the presence of elevated levels of liver enzymes, hemodialysis patients are screened for hepatotropic viral infections: hepatitis B surface antigen, anti-HCV and/

or serum HCV-RNA. Nevertheless, in some patients the etiology of their elevated liver enzymes cannot be established as they are negative for all serological viral markers (including serum HCV-RNA). To investigate whether these hemodialysis patients are affected by an occult HCV infection, 6 Spanish Hemodialysis Units enrolled 42 patients with abnormal liver function tests of unknown etiology[18]. By strand-specific RT-PCR and by *in situ* hybridization it was found that 26/42 (62%) of the patients had HCV-RNA in their PBMC. These patients with occult HCV infection had significantly higher ALT values than the negative ones. In addition, HCV was replicating in the PBMC of 15/26 (58%) of the cases of occult infection. In summary, although these patients are serum HCV-RNA negative they could be potentially infectious as HCV is replicating in their PBMC, thus preventive measures to avoid HCV spread in hemodialysis units must be considered.

ANTIVIRAL TREATMENT IN OCCULT HCV INFECTION

Regarding the possible efficacy of antiviral therapy in occult hepatitis C, Pardo *et al*[19] treated 10 patients with occult HCV infection with pegylated-interferon plus ribavirin for 6 mo, followed by a post-treatment follow-up period of 6 mo. Although all the patients were infected with HCV genotype 1b, they were treated for 6 mo instead of 12 mo (the currently accepted duration of antiviral therapy for patients with chronic hepatitis C and genotype 1b), because they were serum HCV-RNA negative and the percentage of infected hepatocytes is lower than in chronic hepatitis C[14]. At the end of treatment, 80% of the patients had normalized ALT levels and cleared HCV-RNA from PBMC. However, at the end of the post-treatment follow-up only 30% of the patients maintained a complete response (HCV-RNA negative in PBMC and normal ALT levels). Five patients (2 of them were complete responders) underwent a second liver biopsy after treatment and, although none of them lost HCV-RNA in liver, a significant decrease was observed in the amount of intrahepatic viral RNA in comparison to the basal levels. Moreover, in 3 patients liver necroinflammatory activity and fibrosis score had decreased with respect to the pre-treatment histological diagnosis.

It can be concluded that, as reported in chronic hepatitis C (see below), antiviral therapy in occult HCV infection does not lead to a complete eradication of HCV infection, yet it may be useful as liver damage improves. Thus, treatment with pegylated-interferon and ribavirin of patients with occult HCV infection and a stage of liver fibrosis of 2 or more seems advisable.

OTHER FORMS OF OCCULT HCV INFECTION

As commented before, occult HCV infection is characterized by the absence of anti-HCV and of serum HCV-RNA, but viral RNA is detectable in liver and PBMC. However, occult HCV infection may exist in other clinical situations such as in anti-HCV positive patients who are serum HCV-RNA negative and who present normal liver function tests.

One of these populations is the "healthy" HCV-carriers. These patients, who are anti-HCV positive with undetectable serum viral RNA and normal ALT levels, are considered to be subjects who have cleared HCV infection after exposure to HCV. To verify if these patients could have HCV-RNA in their liver, we performed a study with 12 "healthy" anti-HCV carriers[20]. These anti-HCV positive patients were serum HCV-RNA negative and had persistently normal ALT values for a 29 ± 19 mo follow-up period. These patients underwent a programmed interventional laparoscopy and gave their consent for obtaining a liver biopsy specimen during the laparoscopy. The genomic HCV-RNA strand was detected in the liver of 10/12 (85%) of these subjects and it was also found that HCV was replicating in the hepatocytes of the 10 patients in question. All of them were infected by HCV genotype 1b, as demonstrated by sequencing of the HCV genome amplified from the liver. Viral RNA was also found in the PBMC of 6/12 patients and 5 (83%) out of these 6 also had HCV replication in PBMC. Another research group[21] has also demonstrated the presence of HCV infection and replication either in the liver or PBMC of nearly 90% of anti-HCV positive patients with normal ALT values.

Occult HCV infection has also been identified in a similar cohort of patients: those with chronic hepatitis C who have responded to an antiviral therapy with loss of circulating HCV-RNA and normalization of ALT levels. Several papers have reported the presence of an occult HCV infection (persistence of HCV-RNA) in the liver and in PBMC of sustained responders[22-24]. However, other authors have not found viral RNA in these patients[25,26]. These discrepancies could be due to different preservation methods of the liver biopsies, to differences in the sensitivity of the methods employed for HCV-RNA detection, different geographical incidence, *etc*. Thus, further studies are needed to know the real prevalence of occult HCV in complete responder patients. One of the possible consequences of occult HCV infection is the persistence of liver necroinflammation in an important number of sustained responder patients[23,24].

The case of a patient with chronic hepatitis C who cleared serum HCV-RNA with normalization of ALT levels in whom HCV infection reactivated following prednisone therapy after 8.5 years of HCV-RNA negativity has been published. Thus, occult HCV infection should be taken into account when these anti-HCV positive patients with normal ALT levels undergo immunosuppressive therapies[25].

CONCLUSIONS

Occult hepatitis C infection is a new entity that should be taken into account for the diagnosis of patients with a liver disease of unknown origin. Future works should deal with its possible incidence and pathologic relevance in immunosuppressed or HIV coinfected patients, drug abusers or subjects who had received multiple blood transfusions. On the other hand, it would be convenient to perform epidemiological studies on occult HCV infection

among health care staff, patients on hemodialysis, etc to know the prevalence and spread of this infection in these populations. It is also very important to determine the possible incidence and consequences of occult HCV infection in blood donated and transplanted patients.

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REVIEW

Role of ethanol in the regulation of hepatic stellate cell function

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Abstract

Evidence has accumulated to suggest an important role of ethanol and/or its metabolites in the pathogenesis of alcohol-related liver disease. In this review, the fibrogenic effects of ethanol and its metabolites on hepatic stellate cells (HSCs) are discussed. In brief, ethanol interferes with retinoid metabolism and its signaling, induces the release of fibrogenic cytokines such as transforming growth factor β-1 (TGFβ-1) from HSCs, up-regulates the gene expression of collagen I and enhances type I collagen protein production by HSCs. Ethanol further perpetuates an activated HSC phenotype through extracellular matrix remodeling. The underlying pathophysiologic mechanisms by which ethanol exerts these pro-fibrogenic effects on HSCs are reviewed.

Key words: Ethanol; Acetaldehyde; Hepatic stellate cells; liver fibrosis; Type I collagen gene; Transcription factors; Transforming growth factor β-1

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INTRODUCTION

Ethanol abuse is a leading cause for morbidity and mortality throughout the world. It affects many organ systems, most notably the liver causing both acute and chronic liver disease, and the central nervous system[1-3]. Hepatic cirrhosis resulting from alcohol abuse is one of the principal causes of liver-related morbidity and mortality. In the liver, excess ethanol leads to three pathologically distinct disorders, namely fatty liver (alcohol-associated hepatic steatosis), alcoholic hepatitis and cirrhosis. Alcohol-associated hepatic steatosis is the most common form of liver injury and is reversible with abstinence[3-5]. More serious forms of alcoholic liver disease (ALD) include alcoholic hepatitis characterized by persistent inflammation of the liver, and cirrhosis, characterized by progressive hepatic fibrosis. The pathogenesis of ALD is poorly understood, in part because no simple animal model exists that reproduces the full spectrum of the human disease, including the development of cirrhosis[1,4]. In addition, there is considerable variation among individuals in their susceptibility to ALD, so that among people drinking similar amounts, only a proportion develops cirrhosis[1,3-5].

Almost all ingested ethanol is metabolized in the liver. Two major enzyme systems, namely the oxidative and non-oxidative pathways, mediate the initial phase of ethanol metabolism[1,5] (Figure 1). The oxidative pathway comprises the alcohol dehydrogenases (ADH) and members of the cytochrome P450 system (predominantly CYP2E1)[5-7]. This pathway generates acetaldehyde. Acetaldehyde is subsequently metabolized to acetate via the mitochondrial enzyme acetaldehyde dehydrogenase (ALDH). Although acetaldehyde is oxidized to acetate by ALDH, the kinetics of this reaction is sufficiently slow to allow for the accumulation of acetaldehyde in humans or animals consuming alcohol[1,2,5]. The non-oxidative pathway of ethanol metabolism involves the esterification of ethanol with fatty acids to form fatty acid ethyl esters (FAEE), a reaction catalyzed by FAEE synthases[1,5].

Ethanol and its metabolites including acetaldehyde cause liver damage through several interrelated pathways[1,2,8,9]. The oxidation of ethanol is associated with a change in hepatocyte redox homeostasis which can lead to a number of metabolic disorders including lactic acidosis, hyperlipidaemia and hyperuricaemia. Chronic ethanol consumption does not influence ADH activity, but has a profound stimulatory effect on microsomal enzymes, particularly CYP2E1[1,2]. This is in part responsible for the development in alcoholic liver diseases, a rise in oxygen consumption, the excessive production of free radicals and an increase in the metabolism of ethanol, vitamin A and testosterone. Ethanol and acetaldehyde have deleterious effects both direct and indirect, for example by generating reactive oxygen species (ROS) and causing damage to

the intestinal mucosal barrier[1,10]. Cellular oxidative stress that is caused by the relative imbalance between free radical generation and insufficient anti-oxidant defense mechanisms, including reductions in glutathione, vitamin E and phosphatidylcholine, may be a principal mediator for the progression of alcoholic liver disease[1,2,10].

Steatosis, hepatitis and fibrosis seen in persons with ALD are a consequence of complex pathophysiological events involving various cell types within the liver including neutrophils, sinusoidal endothelial cells (SECs), Kupffer cells (KCs), hepatic stellate cells (HSCs) and hepatocytes. Recently, many studies have demonstrated that ethanol and its metabolites including acetaldehyde directly activate HSCs, the principal fibroblastic cell type within the liver[8,9,11]. Ethanol and acetaldehyde directly promote the production of transforming growth factor beta-1 (TGFβ-1) and several extracellular matrix (ECM) constituents including type I collagen by HSCs[8,9,11].

This article reviews recent advances in our knowledge on the effects of ethanol and its metabolites on HSCs.

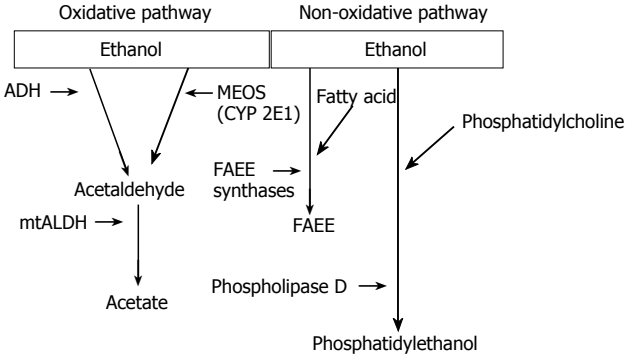

Figure 1 Metabolism of ethanol in the liver via oxidative and non-oxidative pathways. Oxidative pathway: In the first step of oxidation, ethanol is converted to acetaldehyde. Alcohol dehydrogenase (ADH) is the major enzyme. The microsomal ethanol-oxidizing system (MEOS) involves several cytochrome P450 proteins, of which cytochrome P450 2E1 (CYP2E1) is the major constituent. In the second oxidative step, acetaldehyde is rapidly metabolized to acetate by mitochondrial acetaldehyde dehydrogenase (mtALDH). Non-oxidative pathway: The non-oxidative pathway of ethanol metabolism involves the esterification of ethanol with fatty acids to form fatty acid ethyl esters (FAEE), a reaction catalyzed by FAEE synthases. The non-oxidative pathway also generates phosphatidylethanol *via* phospholipase D.

DIRECT EFFECTS OF ETHANOL ON HSCS

A central event in liver fibrosis is the activation of HSCs, which represents a transition from a quiescent vitamin A-rich cell type to a vitamin A-deficient, proliferative, fibrogenic and contractile myofibroblast. Activated HSCs demonstrate altered cell behaviors including proliferation, chemotaxis, fibrogenesis, contractility, matrix degradation, retinoid loss, leukocyte chemotaxis and cytokine release. In total, these changes result in excess ECM deposition which is reabsorbed, culminating in the development of liver fibrosis.

HSCs derived from the intragastric ethanol infusion model of ALD demonstrate an activated phenotype including an increase in collagen I and DNA synthesis[12], expression of α-smooth muscle actin (α-SMA) and depletion of retinyl palmitate[13].

Effect of ethanol on vitamin A metabolism within HSCs

HSCs are the major site of vitamin A storage in healthy adults. Vitamin A in HSCs is in the form of retinyl esters located in cytoplasmic lipid droplets[14]. The three active forms of vitamin A, namely retinol, retinal and retinoic acid (RA) are important regulators of cell proliferation and differentiation, binding to 2 distinct families of ligand-activated transcription factors: the retinoic acid receptor (RARs: RARα, RARβ and RARγ) and the retinoid X receptor (RXR)[15]. The natural ligand for the RARs is all trans-retinoic acid (ATRA). Published data indicate that HSCs from healthy rats express mRNAs in the RARs and RXRs[16].

Nutritionally reduced levels of serum and hepatic vitamin A have been reported in persons with ALD and in animal models of the disease[17,18]. In HSCs, ethanol significantly inhibits RA production[19] and reduces the retinol level[20]. Acetaldehyde exposure results in a reduction in RARβ message and protein in HSCs[21]. There are several other possible mechanisms by which ethanol can interfere with retinoid metabolism in the liver[19], including decreased vitamin A uptake, enhanced degradation of vitamin A in the liver, enhanced vitamin A mobilization from the liver to other organs, and degradation by ethanol of RA into polar inactive metabolites *via* induction of cytochrome P4502E1.

The activation and differentiation of HSCs are characterized by proliferation and an increase in the production of ECM proteins together with a loss of cellular retinoids. Therefore, it is plausible that ethanol-induced RA metabolism in HSCs could play a role in the development of alcohol-related liver fibrosis and cirrhosis.

Ethanol, HSC proliferation and α-SMA expression

Linolenic acid ethyl esters (LAEE), one of the FAEE products of non-oxidative ethanol metabolism, may promote HSC proliferation[22]. This effect is thought to be modulated through increased cyclin E and cyclin-dependant kinase 2 (CDK2) activities[22]. Ethanol, acetaldehyde and lactate, in contrast, have no direct effect on HSC proliferation[23,24].

Ethanol induces early protein expression of α-SMA in cultured HSCs compared to controls[25,26]. Chen and colleagues[27] likewise reported that α-SMA mRNA expression in HSCs is significantly enhanced by exposure to acetaldehyde. However, Poniachik *et al*[24] were unable to replicate this finding. Hence, the effects of ethanol on HSC proliferation and α-SMA expression remain controversial.

Effects of ethanol on ECM production by HSCs

HSC activation is characterized by an increase in the production of ECM, mainly collagen types I and Ⅲ. In addition, HSC activation is associated with alterations in both types of collagen, matrix-degrading metalloproteinases (MMPs) and their inhibitors, tissue inhibitors of metalloproteinases (TIMPs)[28]. Failure of matrix degradation leads to ECM accumulation and progressive hepatic fibrosis[28].

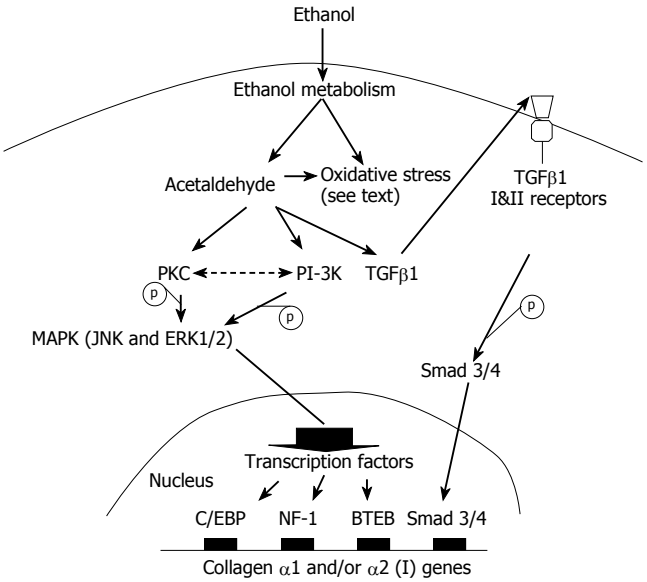

Figure 2 Possible mechanisms for the direct profibrotic effects of ethanol on hepatic stellate cells. Acetaldehyde, together with ethanol or acetaldehyde-derived oxidative stress, induces latent TGFβ1 and TGFβ receptor activation that subsequently leads to Smad3/4 activation and binding to the promoter of collagen I genes. PKC and/or PI-3K kinases are also activated by acetaldehyde. Both kinase systems activate their downstream components, including ERK1/2 and JNK. As a result, C/EBP, NF-1 and/or BTEB transcription factors are activated and therefore up-regulate collagen I gene transcription.

Numerous studies have shown that ethanol and/or its metabolites regulate the expression of multiple components of the ECM in HSCs. Both ethanol and acetaldehyde induce α1 (I) collagen mRNA expression in HSCs[29-33] but not hepatocytes[30]. This effect is protein synthesis-independent[29]. Acetaldehyde increases the steady-stage levels of α2 (I) gene expression[21,34] and the production of type I collagen protein[29-31] in HSCs. Acetaldehyde likewise up-regulates the mRNA expression of MMP-2 and fibronectin in human HSCs[35,36].

Signaling pathways that mediate type I collagen gene transcription in HSCs exposed to ethanol

The mechanisms by which ethanol and its metabolites regulate ECM gene and/or protein expression in HSCs have not been completely elucidated (Figure 2). Several centers have reported that the MAPK and PI-3K pathways are involved[35,37,38]. Anania and colleagues[37] noted that in rat HSCs, phospho-JNK is elevated following exposure to acetaldehyde. Inhibition of JNK by curcumin at low doses reduces acetaldehyde-induced steady-state levels of endogenous α1 (I) collagen mRNA expression[37]. Phosphorylated ERK and p38 are detectable but not significantly elevated. It seems likely therefore that JNK is the principal mediator of acetaldehyde-induced α1 (I) collagen gene up-regulation in rat HSCs. These finding are consistent with those previously reported by Chen *et al*[38]. In contrast, in human HSCs, ERK1/2 and the PI-3K pathway appear to be triggered by acetaldehyde, leading to α2 (I) collagen and fibronectin gene up-regulation[35].

The protein kinase C (PKC) pathway may also play a role in the up-regulation of collagen gene transcription following exposure to ethanol, since PKC is upstream of ERK1/2 and JNK[35,39,40]. Acetaldehyde-elicited α2 (I) collagen and fibronectin gene expression in human HSCs is inhibited by calphostin C (a PKC inhibitor). This PKC inhibitor also reduces the enhancing effect of acetaldehyde on α1 (I) collagen mRNA expression in cultured mouse and human HSCs[35,39]. Other experiments noted that acetaldehyde increases the translocation of PKC activity to membrane fractions[39] and both α1 (I) and α2 (I) collagen gene transcription in a calcium-independent manner[39].

Transcription factors that mediate type I collagen gene expression in response to ethanol

The modulation of gene expression in response to an exogenous or endogenous stimulus occurs through alterations in any one of the steps of gene transcription, mRNA stability, protein translation or protein degradation. Transcription factors are generally classified according to the conserved motifs within either their activation- or DNA- binding domains[41,42]. The binding of transcription factors at DNA-binding sites brings them into proximity with RNA polymerase Ⅱ and components of the transcription complex that assemble in the 5'untranscribed region of genes[42,42]. Transcription factors are then able to exert either a positive or a negative influence on the rate at which the transcription complex transcribes the gene of interest. Transcriptional control of acetaldehyde-induced type I collagen gene expression might be regulated through CCAAT/enhancer-binding proteins (C/EBP), nuclear factor-I (NF-I), basic transcription element binding (BTEB) protein as well as activating protein-1 (AP-1)[34,38,43,44]. The precise mechanisms however, remain to be clarified.

A C/EBP binding site is present in the α1 (I) collagen promoter between -365 and -335 of the transcription start site[44]. Transfection of the α1 (I) collagen promoter mutated at the C/EBP binding site results in unresponsiveness to acetaldehyde, indicating that this site is essential for the collagen gene transcription effect of acetaldehyde[34,44]. C/EBP consists of 6 members. The principal form present in activated HSCs is C/EBPβ[44]. In turn, four C/EBPβ isoforms with approximate molecular weights of 45, 43, 35 and 20 kDa have been identified in activated rat HSCs, with the 35-kDa isoform being predominant[44,45]. Attard *et al*[44] noted that activation of the α1 (I) collagen promoter by acetaldehyde in HSCs is most likely consequent upon an increase in this isoform and increased protein/DNA binding to the C/EBP binding site.

Another report suggests that acetaldehyde-induced α1 (I) gene expression in rat HSCs requires the binding of the acetaldehyde-inducible transcription factor BTEB to a GC box (-1484 to -1476) on the promoter of this gene[43]. In keeping with this proposal, blocking BTEB protein production, results in a reduction in acetaldehyde-induced α1 (I) collagen gene expression[43]. In an extension of the previous report, additional data suggest that acetaldehyde can firstly induce AP-1 activation in HSCs[38,43] and then the activated AP-1 can bind to AP-1 responsive elements in the BTEB promoter to stimulate BTEB expression. The BTEB protein, in turn, stimulates the expression of the α1 (I) gene in HSCs[38,43].

Nuclear factor I (NF-I), a CCAAT binding trans-

cription factor, is also known to bind to and activate the α1 (I) and α2 (I) collagen promoters[46]. Acetaldehyde-induced enhancement of the α2 (I) collagen promoter in activated HSCs is associated with increased binding of NF-I to a consensus consequence located at -352 to -104 bp from the transcriptional start site[34,46,47].

These data suggest that the transcription factors C/EBP, BTEB and NF-1 bind to and activate type I collagen gene transcription through each of them and/or *via* synergic effects, though further characterization of these effects is required. Whether these collagen gene transcription signaling pathways (after exposure to ethanol), are regulated by acetaldehyde itself, or in concert with other profibrogenic mediators such as oxidative stress or TGFβ1 is presently uncertain. The available data are discussed below.

Increased oxidative stress responses and TGFβ1 play an important role in the regulation of type I collagen gene transcription in HSCs

Oxidative stress: Increased oxidative stress is present in the liver after both acute and chronic ethanol administration[48]. Ethanol-induced oxidative stress within hepatocytes can occur acutely through ethanol metabolism or chronically following the induction of CYP2E1[2,10]. The oxidative metabolism of ethanol in hepatocytes elicits a range of mediators including ROS. CYP2E1 in particular has been shown to generate ROS including the superoxide anion, hydrogen peroxide (H_2O_2) and hydroxyethyl free radicals[2,49]. Other sources of free radical generation by ethanol include NADH oxidation by aldehyde oxidase[50].

HSCs contain the enzymes of oxidative ethanol metabolism including ADH and P450 proteins[51-53]. Yamada and Oinonen[52] observed that CYP2E1 is present in rat HSCs as high as 21% of that found in hepatocytes. CYP2E1 is also detectable in the rat hepatic stellate cell line, HSC-T6[51]. In HSC-T6 cells overexpressing ethanol-inducible CYP2E1, time- and dose-dependent induction in collagen α2 (I) mRNA together with increased H_2O_2 production by ethanol has been observed. Antioxidants, including catalase (an H_2O_2 scavenger) prevent this increase in collagen α2 (I) mRNA expression[51]. Because ethanol can be oxidized to acetaldehyde by the peroxidative activity of catalase[54], this decrease in collagen α2 (I) expression by catalase suggests that ethanol-derived acetaldehyde is not responsible for this effect. Svegliati-Baroni *et al*[11] and Greenwel *et al*[35] have also provided evidence to support the concept that increases in mouse α1 (I) and human α2 (I) collagen gene expression in HSCs by acetaldehyde are linked to elevated H_2O_2 production. For example, acetaldehyde-elicited type I collagen gene expression can be blocked by the addition of catalase[11,35], and is in part, TGFβ1-independent[11]. It is known that H_2O_2 activates MAPK pathways[49] and this activity might enhance the binding of the down stream transcription factors to acetaldehyde-responsive elements within the type I collagen promoter. Likewise, leptin induces H_2O_2 production and contributes to TIMP-1 expression in HSCs[55]. Collectively, these data suggest that increased H_2O_2 generation during the metabolism of ethanol by HSCs might play a critical role in their activation.

TGFβ-1: Ethanol and acetaldehyde increase autocrine TGFβ1 expression in HSCs. In turn, TGFβ1 is able to up-regulate type I collagen gene expression[32,34,38]. Anania and colleagues[34] noted that the effects of acetaldehyde-induced TGFβ1 in the regulation of α2 (I) collagen gene expression are mediated by a factor or factors that bind to nuclear factor I (NF-I) consensus sequence located at the -352 to -104 region of the α2 (I) gene promoter. Acetaldehyde further increases the secretion of both latent and active forms of TGFβ1 in cultured rat HSCs[38], and induces the expression of the type Ⅱ TGFβ receptor which is required for all TGFβ-mediated signaling events[38]. In transient transfection experiments, the combination of TGFβ1 and acetaldehyde could result in greater activation of the mouse α2 (I) collagen promoter than either TGFβ1 or acetaldehyde alone[34]. Taken together, these observations suggest that TGFβ1 could play a key role in acetaldehyde-induced collagen I gene activation.

Chen *et al*[38,43] have noted that acetaldehyde stimulates latent TGFβ1 secretion and TGFβ type Ⅱ receptor gene expression. BTEB might be the principal transcription factor binding to the GC box of the type Ⅱ TGFβ receptor gene promoter[38]. The authors proposed a model wherein acetaldehyde activates signal transduction pathways including PKC, JNK and ERK, leading to activation of AP-1. AP-1 is proposed to activate the gene expression of BTEB. BTEB then up-regulates TGFβ type Ⅱ receptor gene expression in HSCs. By stimulating latent TGFβ1 activation and secretion, as well as up-regulating the expression of TGFβ type Ⅱ receptor, acetaldehyde activates TGFβ1 signaling, which eventually enhances expression of the α1 (I) collagen gene in HSCs[38].

The precise molecular mechanisms by which acetaldehyde elicits TGFβ1 production in HSCs are largely unknown. Acetaldehyde might directly bind to the TGFβ1 gene promoter leading to its activation. Alternatively, acetaldehyde might bind to other gene promoters of transcription factors that in turn activate the TGFβ1 gene.

OTHER DIRECT FIBROGENIC EFFECTS OF ETHANOL ON HSCS

Rodriguez-Fragoso and his colleagues[56] investigated the effects of the activity of urokinase type plasminogen activator (uPA) in the CFSC-2G stellate cell line and demonstrated that acetaldehyde (175, 250 and 350 μmol/L) enhances uPA gene expression. This is accompanied with a concomitant increase in production of type I collagen. uPA plays an important role in matrix remodeling under a wide range of physiological and pathological conditions, activates TGFβ1 and induces proliferation of HSCs[57,58]. Furthermore, profibrogenic mediators including IL-6, TNF-α, malondialdehyde (MDA) and intracellular GSSG have been reported to increase in CFSC-2G cells treated with ethanol or acetaldehyde[59-61].

Malondialdehyde-acetaldehyde (MAA) -protein adducts induce a dose- and time-dependent increase in the secretion of chemokines including monocyte chemoattractant protein (MCP)-1 and macrophage inflammatory protein (MIP)-2 as well as an increase in

the production and expression of intercellular adhesion molecule-1 (ICAM-1) in activated rat HSCs[62,63]. These effects may contribute further to the activation of HSCs and the subsequent development of alcohol-associated liver fibrosis.

ACTIVITY OF ETHANOL/ACETALDEHYDE ON PANCREATIC STELLATE CELLS

Rat pancreatic stellate cells (PSCs) exhibit features similar to those of HSCs[64-66]. These cells are abundant in alcoholic chronic pancreatitis in humans, suggesting a central role of this cell type in pancreatic fibrosis[65]. An effect of ethanol on the modulation of PSCs has been documented. Ethanol and acetaldehyde increase α-SMA protein and type I collagen synthesis in PSCs[67], likewise enhance PSC MMP-2 and TIMP-2 gene expression as well as TIMP-2 protein secretion[67-69]. Both ethanol and acetaldehyde increase the activation of all 3 subfamilies (ERK1/2, JNK/SAPK and p38 kinase) of the MAPK pathway in PSCs. Only p38 MAPK is responsible however, for the induction of α-SMA and α1 (I) collagen gene expression[70]. Moreover, ethanol and acetaldehyde-induced MAPK activation can be blocked by the antioxidant N-acetyl-cysteine, suggesting a role of oxidative stress in signal transduction[68,71].

CONCLUSION

Ethanol can be metabolized in hepatocytes and stellate cells to generate acetaldehyde and other metabolites. Ethanol and/or its metabolites including acetaldehyde have direct effects on HSC activation. These effects might be mediated by ethanol/acetaldehyde and/or ethanol/acetaldehyde-induced oxidative stress and TGFβ1 expression which activate relevant signaling pathways leading to the binding of transcription factors to the type I collagen gene promoter (Figure 2). As a result, ethanol augments the production of extracellular matrix proteins. Ethanol also stimulates the production of other profibrotic mediators, including IL-6, TNF-α and uPA. Taken together, these effects of ethanol/acetaldehyde on HSCs play an important role in the development of alcohol-associated liver fibrosis. Characterization of the key genes initiating and perpetuating the process of HSC activation by ethanol helps to further elucidate the molecular mechanisms of alcohol-associated liver fibrosis. In the future, it is hoped that specific, directed pharmacological agents can be selected and/or developed that target these mechanisms and thereby prevent or retard the fibrogenesis induced by alcohol.

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REVIEW

Methylation in esophageal carcinogenesis

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Abstract

Genetic abnormalities of proto-oncogenes and tumor suppressor genes have been demonstrated to be changes that are frequently involved in esophageal cancer pathogenesis. However, hypermethylation of CpG islands, an epigenetic event, is coming more and more into focus in carcinogenesis of the esophagus. Recent studies have proved that promoter hypermethylation of tumor suppressor genes is frequently observed in esophageal carcinomas and seems to play an important role in the pathogenesis of this tumor type. In this review, we will discuss current research on genes that are hypermethylated in human esophageal cancer and precancerous lesions of the esophagus. We will also discuss the potential use of hypermethylated genes as targets for detection, prognosis and treatment of esophageal cancer.

Key words: Methylation; Esophageal cancer; Tumor suppressor gene; Carcinogenesis

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INTRODUCTION

Esophageal cancer is one of the least studied and deadliest cancers, with a remarkable geographical distribution and a low likelihood of cure[1]. Therefore, the current challenges in the management of esophageal cancer are to obtain a better understanding of the underlying molecular biological alterations to provide new treatment options. Cancer of the esophagus exists in two main forms with different etiological and pathological characteristics-esophageal squamous cell carcinoma (ESCC) and esophageal adenocarcinoma (EAC)[1]. It is well known that esophageal carcinogenesis is a multistage and progressive process which includes basal cell hyperplasia (BCH), dysplasia (DYS), carcinoma in site (CIS) and advanced esophageal carcinoma[1,2]. A variety of genetic lesions are involved in esophageal carcinogenesis, including gene amplifications, loss of heterozygosity(LOH) or homozygous deletions, mutations, and chromosomal rearrangements[1,2]. From the above mentioned genetic lesions , mutations are greatly focused on. The vast majority of esophageal cancers have mutations of the TP53 and p16 genes at an early stage followed by mutations in genes such as APC, Rb and cyclin D_1 at later stages of progression[3]. Furthermore, the alteration of the gene mutations may show different types in ESCCs and EACs. For example, ESCCs and EACs show distinct patterns of TP53 mutations, namely a high prevalence of G > A transitions at CpG sites in EACs whereas in ESCCs a higher prevalence of G to T transversions and mutations at A:T base pairs is present[4].

Genetic mutation of genes that inhibit the formation of tumors has long been known to be one of the main driving forces in the development of cancer[1]. However, recent data have focused our attention to the contribution of epigenetics to tumorigenesis. In tumorigenesis of the esophagus, the epigenetic inactivation of genes is as an important a driving force as the inactivation of genes by mutation[5]. 'Epigenetic' events, i.e. heritable changes in gene function which cannot be explained by changes in DNA sequence, are composed of histone acetylation, the chromatin structure and DNA methylation[5]. DNA methylation seems to be the most important mechanism for "epigenetic change" at present[5,6]. Through a process of post-replicative covalent modification catalysed by DNA methyltransferases(DNMTs), the DNA of mammalian cells contains a 'fifth base', namely 5-methylcytosine. The most frequent target for this modification is cytosine in the context of the dinucleotide CpG[5]. Throughout the genome CpG dinucleotides are found at one-fifth of their predicted frequency[6]. In marked contrast to the genome-wide underrepresentation of CpGs, there are regions of the genome termed CpG islands which have maintained their expected frequency of the dinucleotides. And the CpG islands are often found within the promoter of genes[6,7]. It has been known for many years that, in general terms, there is an inverse relationship between the density of promoter methylation and the transcriptional activity of a gene[8,9]. The mechanism of gene silencing by promoter hypermethylation has recently been shown to be related to the recruitment of repressor protein complex, resulting in

de-acetylation of the chromatin and histone, thus barring access to the active transcription complex. However, the actual mechanisms by which DNA methylation modulates gene expression have remained elusive[7,10]. The assays for detection of cytosine methylation can be divided into two groups: restriction enzyme-based and bisulfite treatment-based[11,12]. The former employs the inhibition of certain restriction enzymes by methylation of their recognition sites as an indicator for the presence of methylation. The latter translates the epigenetic information of cytosine methylation in primary sequence differences by converting unmethylated cytosine to uracil whereas methylated cytosine remains unaltered. The bisulfite-converted genomic DNA can be analyzed by a wide variety of PCR-based methods[11,12]. Methylation is needed for the normal development of cells. And genome stability and normal gene expression are largely maintained by a fixed and predetermined pattern of DNA methylation[13]. Aberrant methylation confers a selective growth advantage that results in cancerous growth[13]. From various lines of evidence, it is known that the methylation pattern of the cancerous cell is associated with a broad genomic hypomethylated state that is often accompanied by a more regional and locus-specific hypermethylated pattern[7]. The presence of alterations in the profile of DNA methylation in cancer was initially thought to be exclusively a global hypomethylation of the genome that would possibly lead to massive overexpression of oncogenes whose CpG islands were normally hypermethylated[14]. Nowadays, however, this is considered to be an unlikely or, at least, incomplete scenario. The popularity of the concept of demethylation of oncogenes leading to their activation is in clear decadency[14,15]. Hypermethylation of CpG islands located in the promoter regions of tumor suppressor genes(TSGs) is now firmly established as an important mechanism for gene inactivation[16,17,18]. The particular genes that are hypermethylated in tumor cells are strongly specific to the tissue of origin of the tumor. A profile of CpG island hypermethylation exists according to the tumor type[15]. The mechanism responsible for this type of pattern remains largely unclear. Moreover, accumulating evidence indicates that CpG island hypermethylation is an early event in cancer development and, in some cases, may precede the neoplastic process[19]. Therefore, such profiles would provide invaluable insight into mechanisms underlying the evolution of each tumor type and will provide new molecular markers. This review will focus on the current understanding of DNA methylation abnormalities in esophageal cancer and discuss how this knowledge contributes to our understanding of the pathogenesis of esophageal cancer.

STUDIES OF GENE PROMOTER HYPERMETHYLATION IN ESOPHAGEAL CANCER

Putative tumor suppressor genes, involving apoptosis, cell adherence, DNA repair, and the cell cycle, have been investigated for hypermethylation by various techniques in esophageal cancer. Below, we have chosen to describe those genes that have been most extensively studied in the past and that have been shown to undergo epigenetic changes in esophageal carcinoma.

p14^{ARF}, p15 and p16

The 9p21 chromosomal band is one of the most frequently altered genomic regions in human cancers[20]. Within a short distance of 50 kb, a gene cluster consisting of three genes, p14^{ARF}, p15 and p16, is harbored. All of which have putative tumor suppressor roles[20,21,22]. Inactivation of p14^{ARF}, p15 and p16 genes has been observed in many types of human cancers including ESCC[21,22,23]. For example, the results from immunohistochemical analysis indicated that p16 expression was present in only 3 out of 22 ESCC cases[24]. Some studies showed that germline mutations in the p16 gene might be related to familial melanoma[25], but another study found the mutation of the p16 gene in esophageal cancer was rare[26]. Hemizygous and homozygous deletion at 9p21 are widely considered to be one of the primary mechanisms of p16/p15 inactivation[26]. Recently, however, aberrant methylation of the CpG islands at the promoter regions of p16 and p15 genes was reported in many cancers and was associated with loss of transcription[8,27]. Abbaszadegan *et al*[28] assessed a large family with clustering of ESCC in northeastern Iran and found aberrant p16 promoter methylation in 64% of ESCC family members and none in normal volunteers. By analyzing the p14^{ARF}, p15, and p16 genes individually in 40 ESCCs, Xing *et al*[29] detected aberrant promoter methylation of the p16 gene in 40%, of p14^{ARF} in 15%, and of p15 in 13% tumor samples. They further detected homozygous deletion of p16 in 18%, of p14^{ARF} in 33%, and of p15 in 40% tumor samples, and detected no mutation in the p14^{ARF} and p16 genes[29]. Hardie *et al*[30] reported that hypermethylation of the p16 promoter is detected in 85% (18/21) of EACs while p16 mutations are uncommon (1.9%; 1/54). Another report found that in 50 Barrett's esophagus-associated EACs, hypermethylation of p16 was present frequently (54%), but hypermethylation of p14^{ARF} was absent[27]. The above results suggest that p14^{ARF}, together with p15, is a primary target of homozygous deletion, whereas p16 is the hypermethylation hotspot in human esophageal cancer.

The FHIT gene

The FHIT gene is located at chromosome 3p14.2 and encodes a polypeptide of 147 amino acids[31]. FHIT allelic deletions and reduced or absent FHIT protein expression have been observed in a variety of tumors suggesting a putative tumor suppressor function[31,32]. In ESCCs, the CpG island in the FHIT promoter region was hypermethylated in 25 of the 36 (69%) analyzed cases, significantly correlated with the deletion of FHIT protein expression[33]. Methylated ESCC cell lines exhibit re-expression of the FHIT gene and demethylation in the CpG islands after treatment with demethylating agent 5-aza-2'-deoxycytidine[34]. Another report showed that aberrant methylation of FHIT was found in 85 of 257 (33%) ESCCs[35]. These findings suggest that methylation of the 5' CpG islands of the FHIT gene is closely

associated with transcriptional inactivation and might be involved in tumor development of the esophagus.

The RARβ2 gene

The retinoic acid receptor-beta2 (RARβ2) gene located at 3p24 has been intensively studied in many cancers and found to have defective function, thus making it a candidate TSG[36]. RARβ2 expression was detected in 88% (14/16) of normal esophageal tissues and only 54% (84/162) of esophageal carcinomas[36]. And 14 of 20 (70%) ESCC samples had hypermethylation of the RARβ2 promoter[37]. Another group reported that 34 of 47 (73%) primary resected ESCC samples showed RARβ2 methylation[38]. After 5-aza-2'-deoxycytidine treatment the expression of RARβ2 was reversed in two RARβ2-downregulated ESCC cell lines[39]. These results identified methylation as the underlying mechanism for this frequent loss of RARβ2 in esophageal cancer.

The APC gene

The adenomatous polyposis coli (APC) gene, located on chromosome 5q21, is a TSG in the wnt signaling pathway[40]. APC shows frequent LOH in esophageal carcinomas, and the prevalence of mutations in the APC gene in esophageal carcinomas is low[41]. Hypermethylation of the promoter region of the APC gene occurred in abnormal esophageal tissue in 48 of 52 (92%) patients with EAC, in 16 of 32 (50%) patients with ESCC, but not in matching normal esophageal tissues[41]. So methylation of the promoter region of this gene constitutes an alternative mechanism of gene inactivation in esophageal carcinoma.

The ER gene

The estrogen receptor (ER) gene at chromosome 6q, which has growth and metastasis suppressor activity in many different cell types, is widely expressed in tissues other than breast, and is methylated in 51% of EAC patients[42].

The MGMT gene

The human enzyme O^6-methylguanine-DNA methyltransferase (MGMT) is located on chromosome band 10q26, and protects the cell from guanine methylation by irreversibly transferring the alkyl group of the O^6-methylguanine to a specific cysteine residue within the molecule[43]. Approximately 20% of tumor cell lines lack MGMT activity and are highly sensitive to alkylating agents[44]. In established cancer cell lines, MGMT expression appears to be correlated with methylation in the promoter of the gene[44]. The gene has been shown to be methylated in 46/119 (39%) ESCC, but all 21 normal esophageal tissues had unmethylated MGMT[45]. Another report studied the role of DNA hypermethylation in the loss of expression of MGMT during the development of ESCC, and found that 5 of 17 (29%) normal esophagus, 10 of 20 (50%) BCH, 8 of 12 (67%) DYS, and 13 of 18 (72%) ESCC samples had DNA hypermethylation in the MGMT promoter region, showing a gradual increase with the progression of carcinogenesis, while the frequency of the loss of MGMT mRNA and protein expression progressively decreased from normal to BCH, DYS, and ESCC, and it was highly correlated with MGMT promoter hypermethylation[46].

The E-cadherin gene

E-cadherin gene on chromosome 16q22.1 encodes a Mr 120 000 transmembrane glycoprotein expressed on the surface of epithelial cells. In epithelial tissues, E-cadherin mediates homophilic, Ca^{2+}-dependent intercellular adhesion that is essential for the maintenance of normal tissue architecture[47]. Loss of E-cadherin expression occurs in a variety of human tumors and is correlated with invasion and metastasis, and activation of E-cadherin results in the growth inhibition of tumor cell lines[48]. E-cadherin can be targeted by both genetic and epigenetic means. Moreover, the hypermethylation of E-cadherin was seen frequently in most tumor types, but mutations only frequently in a small number of specific subtypes[48]. In esophageal carcinoma, downregulation of E-cadherin is common and is associated with an increase in invasive and metastatic potential, but mutations of the gene are rare[49]. E-cadherin was methylated in 26 of 31 (84%) EAC specimens, 16 of 20 (80%) ESCC samples and 4 of 6 ESCC cell lines[49,50,51]. And treatment of E-cadherin-negative carcinoma cells with the demethylating agent, 5-aza-2'-deoxycytidine, induced re-expression of the gene[51]. These data suggest that epigenetic silencing via aberrant methylation of the E-cadherin promoter is the critical mechanism for inactivation of this gene in esophageal cancer.

The TSLC1 gene

The TSLC1 (tumor suppressor in lung cancer) gene located on 11q23.2 was first characterized as a TSG in human non-small cell lung cancer(NSCLC) and termed TSLC1[52]. The tumor suppressor role of this gene has been demonstrated in the cell lines of NSCLC, hepatocellular carcinoma, pancreatic cancer and ESCC[52,53]. Loss of TSLC1 expression was observed in 75% of the ESCC cell lines and 50% of the primary tumors from ESCC patients[53]. Ito *et al*[53] examined the methylation status of six cytosine residues of CpG sites in a putative promoter sequence upstream from the TSLC1 translation initiation site by bisulfite sequencing in four cell lines, including KYSE270, which expressed TSLC1, and KYSE410, KYSE520, and KYSE960, which did not express it. They also found all of the cytosine residues in KYSE270 DNA were unmethylated, whereas all of the six cytosine residues in KYSE520 DNA and five residues in KYSE410 and KYSE960 DNA were methylated. Especially, the cytosine residues in KYSE520 DNA were all hypermethylated. However, the report about the status of the promoter methylation of TSLC1 gene in esophageal cancer tissue has not been available.

The RASSF1A gene

Many known RAS effectors are oncoproteins on their own. Less is known about Ras effectors possessing tumor suppressor properties[54]. Recently, a new family of genes

encoding a putative Ras effector, the Ras-association domain family 1 (RASSF1) gene, has been identified within the critical lung and breast cancer deletion region at 3p21.3. The RASSF1 locus encodes several major transcripts by alternative promoter selection and alternative mRNA splicing: RASSF1A, RASSF1B and RASSF1C. Many studies have suggested that RASSF1A was a new putative TSG[54,55]. RASSF1A acts as a negative effector of Ras in a pro-apoptotic signaling pathway. Interestingly, mutational inactivation of this gene is very rare (< 2%), and the main mechanism of its inactivation is through promoter methylation and LOH[56]. The RASSF1A isoform is highly epigenetically inactivated in lung, breast, ovarian, kidney, prostate, thyroid, esophagus and several other carcinomas[54]. Hypermethylation of RASSF1A was detected in 73% of ESCC cell lines and 51% of primary ESCCs, whereas only 4.3% of RASSF1A hypermethylation were detected in corresponding noncancerous tissues[38]. There was a statistically significant correlation between the presence of hypermethylation and tumor stage[57]. Wong *et al*[58] also found that RASSF1A was partially methylated in 3/7 (43%) esophageal cancer cell lines; 22/64 (34%) ESCCs and 3/64 (4.7%) corresponding non-tumor samples; and was not methylated in 2 immortalized normal oesophageal epithelial cell lines and 6 normal esophageal epithelium samples. These findings suggest that epigenetic silencing of RASSF1A gene expression by promoter hypermethylation could play an important role in ESCC carcinogenesis.

Besides the above mentioned genes, there are hypermethylations of some other genes involving esophageal cancer, including hMLH1[59,60], VHL[38], TIMP3[42,61], DAP-kinase[42], pRb[62], ECRG4[63], Chfr[64], HLA class I[65], EYA4[66], CDH13[67], SFRP1[68] and PGP9.5[69]. Table 1 gives a summary of the profile of gene hypermethylation in human esophageal cancer.

From the above mentioned reports, we find that there is obvious different methylation frequency in a gene from different authors in some cases, which may be due to different assay methods and different specimen resources. And the geographical difference may be due to variable carcinogens in the different areas. Furthermore, there seems to be obvious difference of methylation frequency of some genes between ESCCs and EACs. For example, hypermethylation of the promoter region of the APC gene occurred in 48 of 52 (92%) patients with EAC, but in 16 of 32 (50%) patients with ESCC[41], which suggests that hypermethylation of the APC gene has distinct roles in ESCC and EAC.

Table 1 Compilation of genes hypermethylated in esophageal cancer *n* (%)

Gene	Entity of pathology	Incidence of methylation		Reference
		Cancer tissue	(Adjacent) nonmalignant tissue	
$p14^{ARF}$	ESCC	6/40 (15)	ND	29
	EAC	0/50 (0.0)	ND	27
p15	ESCC	6/34 (18)	ND	26
	ESCC	5/40 (13)	ND	29
p16	ESCC	17/34 (50)	ND	26
	ESCC	18/28 (64)	0/30 (0.0)	28
	ESCC	16/40 (40)	ND	29
	EAC	27/50 (54)	ND	27
	EAC	8/21 (38)	ND	27
	EAC	16/41 (39)	10/41 (24)	42
FHIT	ESCC	25/36 (69)	ND	33
	ESCC	85/257 (33)	ND	35
$RAR\beta_2$	ESCC	14/20 (70)	2/17 (12)	37
	ESCC	34/47 (73)	18/47 (38)	38
APC	EAC	48/52 (92)	0/52 (0.0)	41
	ESCC	16/32 (50)	0/32 (0.0)	41
	EAC	28/41 (68)	3/41 (7.3)	42
	EAC	39/50 (78)	ND	27
ER	EAC	21/41 (51)	5/41 (12)	42
MGMT	ESCC	46/119 (39)	0/21 (0.0)	45
	EAC	24/41 (56)	10/41 (24)	42
E-Cadherin	EAC	26/31 (84)	ND	49
	EAC	27/41 (66)	5/41 (12)	42
	ESCC	16/20 (80)	ND	50
TSLC1	ESCC	28/56 (50)	ND	53
RASSF1A	ESCC	24/47 (51)	2/47 (4.3)	38
	ESCC	22/64 (34)	3/64 (4.7)	58
hMLH1	ESCC	0/30 (0.0)	ND	59
	ESCC + EAC	79/124 (64)	ND	60
VHL	ESCC	6/47 (13)	0/47 (0)	39
TIMP3	EAC	8/41 (19)	0/41 (0.0)	42
	EAC	71/79 (90)	0/79 (0.0)	61
DAP-kinase	EAC	8/41 (19)	2/41 (4.9)	42
pRb	EAC	10/30 (33)	ND	62
ECRG4	ESCC	12/15 (80)	3/20 (15)	63
Chfr	ESCC + EAC	7/43 (16)	ND	64
HLA class I	ESCC	13/29 (45)	ND	65
EYA4	EAC	33/40 (83)	2/58 (3.4)	66
CDH13	ESCC+EAC	5/37 (14)	ND	67
SFRP1	EAC	37/40 (93)	3/30 (10)	68
PGP9.5	ESCC	21/50 (42)	ND	69

ND: Not done.

METHYLATION IN SERUM DNA FROM ESOPHAGEAL CANCER PATIENTS

Despite advances in diagnosis and treatment of various cancers, early detection and treatment of cancer remain a challenge. One potential early detection biomarker is DNA methylation of the promoter region of certain cancer-associated genes[70]. Genetic analysis has shown that cell-free circulating DNA in plasma or serum of cancer patients shares similar genetic alterations to those described in the corresponding tumor[70,71]. Numerous studies have demonstrated the presence of promoter hypermethylation of tumor suppressor genes in the serum DNA of patients with various cancers[72]. Hypermethylated APC DNA was observed in the plasma of 13 of 52 (25%) EACs and 2 of 32 (6.3%) ESCCs[41]. Hibi *et al*[73] found that aberrant promoter methylation of the p16 gene was detected in 31 of 38 (82%) ESCCs, and 7 of these 31 (23%) patients with a p16 alteration in the primary tumor had the same methylation changes in the corresponding serum DNA. This study yielded a promising result: a tumor associated DNA alteration could be detected in the serum of 18% of ESCC patients (7 of 38 patients) using p16 methylation as a target. Moreover, the clinical sensitivity of this assay can be potentially improved by incorporating other possibly methylated target genes, which have been

estimated in other tumor types. For example, Esteller *et al*[74] analyzed primary NSCLCs and serum from 22 patients for the methylation pattern of four TSGs (DAPK, GSTP1, p16, and MGMT). Methylation of at least one of these genes was detected in 68% of NSCLCs. Comparing primary tumors with methylation and matched serum samples, 73% of the matched serum samples were found to be methylated[74]. In addition, none of the sera from patients with tumors not demonstrating methylation were positive[74]. Therefore, combined detection of aberrant promoter hypermethylation of cancer-related genes in serum may be useful for esophageal cancer diagnosis or the detection of recurrence.

PROGNOSTIC SIGNIFICANCE OF GENE HYPERMETHYLATION

In the past few years, numerous attempts have been made to establish a genetic technique for reliably predicting tumor prognosis, but these attempts have been hindered by two main problems. First, only a few genes are somatically mutated in solid tumors and, second, because cell populations of primary neoplasms are heterogeneous, no single marker can accurately predict the behavior of the tumor[13]. Fortunately, emerging evidence suggests a possible prognostic value of gene promoter hypermethylation[75]. Lee *et al*[35] reported that aberrant methylation of the FHIT promoter in ESCC was found to be significantly associated with a poor prognosis for stage 1-2 cases. Mandelker *et al*[69] reported that PGP9.5 methylation was an independent prognostic factor for ESCC survival ($P = 0.03$) . Kawakami *et al*[41] reported that high plasma levels of methylated APC DNA were statistically significantly associated with reduced EAC patient survival. Brock *et al*[42] analyzed the methylation status of seven genes (including APC, E-cadherin, MGMT, ER, p16, DAP-kinase and TIMP3) of 41 EAC samples and found that DNA methylation of some genes individually showed only trends toward diminished survival, whereas patients whose tumors had > 50% of their gene profile methylated had both significantly poorer survival and earlier tumor recurrence than those without positive methylation. The data suggest that combined detection of methylation status for multiple genes is an effective strategy for prediction of esophageal tumor behavior. Although some genes that are frequently inactivated by methylation and are of prognostic impact for esophageal cancer patients have already been found, additional genes need to be identified. Thus, patients with a worse prognosis could be selected. These patients might benefit from a more aggressive treatment strategy.

ABERRANT DNA METHYLATION IS AN EARLY EVENT IN ESOPHAGEAL CARCINOGENESIS

In many tumors, it has been proved that aberrant DNA methylation frequently occurs in precancerous tissue as well as cancer tissue, and both factors, genetic and epigenetic, lie at the origin of carcinogenesis[76]. The relative contribution of each varies significantly in different human tumors[76]. Nie *et al*[77] compared hypermethylation of p16, p15, p14, HLA-A, -B, -C, hMLH1, E-cadherin, FHIT and VHL genes in precancerous esophageal tissues and found that in 48 biopsy samples with BCH or DYS, the most frequent hypermethylated genes were p16 (19%) and $p14^{ARF}$ (15%), and seventeen out of these 48 samples (35%) contained hypermethylation of at least one gene. In the resected tissues, 52% of the BCH and 81% of the tumors showed hypermethylation of at least one gene. Another study reported that 2 of 17 (12%) normal esophagus, 9 of 21 (43%)BCH, 7 of 12 (58%) DYS, and 14 of 20 (70%) ESCC samples had hypermethylation of the RARβ_2 promoter region[37]. As to progression of EAC, it has been reported that, methylation of the p16 promoter was detected in 18 of 22 (82%) EAC and 10 of 33 (30%) premalignant lesions, whereas no methylation of the p16 promoter was found in normal esophageal epithelia[78]. Hardie *et al*[30] reported hypermethylation of the p16 promoter was detected in 77% (14/18) of Barrett's epithelia, and in 85% (18/21) of EACs. These data suggest that aberrant DNA methylation participates early in the development of esophageal cancer. Recently, the lab of professor Yang CS reported that EGCG, the major polyphenol from green tea, inhibited DNMT activity and reactivated several methylation-silenced genes, including p16, RARβ_2, MGMT and hMLH1, in human esophageal cancer KYSE 510 cells, accompanied by the expression of mRNA of these genes[79]. The result suggests that methylation might be a new target of chemopreventive activity. In the last two decades, it has been proven that many drugs, such as tamoxifen, aspirin, COX-2 inhibitors, possess positive chemopreventive activity against esophageal cancer[2]. However, the exact mechanisms have not been elucidated so far. Therefore, it will be very attractive to examine the effect of these drugs on promoter methylation status of key genes in esophageal cancer cells, esophageal cancer tissue, and especially precancerous tissue of the esophagus.

HYPERMETHYLATION AS A TARGET OF THERAPEUTIC INTERVENTION

It has been reported that demethylating agents 5-azacytidine and 5-aza-2'-deoxycytidine can restore the normal demethylated state of several types of TSGs and increase their expression in various cancers, including esophageal cancer, *in vitro* and *in vivo*[13,14,15,39,54]. Since methylation and transcriptional status are inversely correlated, the use of demethylating agents appears to be a promising option for the treatment of tumors. Methylation of genes in tumor cells could provide a tumor-specific target for new therapies[80,81,82]. In fact, these demethylating agents have exhibited significant activity in the treatment of patients with myelodysplastic syndrome, chronic myeloid leukaemia and acute myeloid leukaemia[83,84]. However, preliminary experience with these agents in solid tumors has been relatively low[85]. Esophageal tumor shows a high prevalence of TSG hypermethylation, and the above studies demonstrated that gene expression could be restored after treatment of esophageal tumor cells

with demethylating agents *in vitro*. However, up to date the clinical trial about demethylating agents in esophageal cancer is unavailable. Although it is too early to make any expectation about the effect of these drugs on esophageal cancer, this is a very promising concept and needs to be tested in clinical trials.

CONCLUSION AND PERSPECTIVES

Esophageal carcinogenesis is a stepwise process of the accumulation of genetic and epigenetic abnormalities. It has become clear that promoter hypermethylation of TSGs is as important for this multistep process as genetic changes in the progression of esophageal carcinogenesis. The steadily growing list of genes inactivated by promoter hypermethylation in esophageal carcinoma provides not only new insights into the molecular basis of the diseases but also a long list of interesting candidate genes for the development of molecular markers which might contribute to the improvement of diagnosis and also prognosis. In addition, the fact that methylation can be reversed *in vitro* and the effect of the demethylating agent 5-aza-2'-deoxycytidine *in vitro* raise hope for new treatment strategies for esophageal cancer patients. Furthermore, understanding of the significance of aberrant DNA methylation in the precancerous stage may show that a new strategy, correction of aberrant DNA methylation, can prevent esophageal cancer in people with premalignant lesions, such as Barrett's esophagus, BCH and DYS.

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REVIEW

Cancer gene therapy targeting angiogenesis: An updated review

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Abstract

Since the relationship between angiogenesis and tumor growth was established by Folkman in 1971, scientists have made efforts exploring the possibilities in treating cancer by targeting angiogenesis. Inhibition of angiogenesis growth factors and administration of angiogenesis inhibitors are the basics of anti-angiogenesis therapy. Transfer of anti-angiogenesis genes has received attention recently not only because of the advancement of recombinant vectors, but also because of the localized and sustained expression of therapeutic gene product inside the tumor after gene transfer. This review provides the up-to-date information about the strategies and the vectors studied in the field of anti-angiogenesis cancer gene therapy.

Key words: Anti-angiogenesis; Tumor growth; Cancer gene therapy

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INTRODUCTION

Angiogenesis is the formation of new blood vessels from pre-existing ones. Many developmental and pathological processes require angiogenesis[1]. As proposed by Folkman in 1971, angiogenesis is required for tumor growth[2]. Angiogenesis consists of several steps: endothelial cell (EC) proliferation, migration, basement membrane degradation, and new lumen organization[3]. This multi-step process is determined by a net balance between pro- and anti-angiogenesis regulators in the circulation blood, which are released from activated ECs, monocytes, smooth muscle cells and platelets[3].

The growth of tumor depends on new blood vessel growth and involves three steps: angiogenesis, vasculogenesis and intussusception[4]. Without angiogenesis, a solid tumor rarely grows larger than 2 to 3 mm[5]. As shown in Figure 1, ECs and tumor cells release angiogenesis regulators like vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF) and transforming growth factor (TGF) to mediate angiogenesis. The result is the development of invasive tumor. In addition to the presence of angiogenesis factors, activation of oncogene and loss of tumor suppressor gene are also essential for an angiogenesis phenotype that supports tumorigenicity[6]. As a result, anti-angiogenesis has been regarded as a target for cancer therapy.

There are already several extensive reviews on the development of anti-angiogenesis cancer gene therapy[3,7-10]. In the 2001 review, Liau *et al*[7] compared and contrasted the gene approach and recombinant protein approach. In the editorial written by Lau and Bicknel[9], the authors compared the delivery of the genes of anti-angiogenic factors with that of the therapeutic proteins. They suggested that the delivery of genes can allow a high local expression of the protein at the sites of active tumor growth[9]. El-Aneed pointed out in his review, which summarizes the strategies in cancer gene therapy, that the ease of accessing ECs of the blood vessels is one of the main advantages of gene delivery approach[10]. Figure 2 shows that the delivery of the anti-angiogenesis gene into tumor cells or ECs can inhibit tube formation, EC migration and proliferation. This can result in tumor necrosis. In this review, updated information on the development of cancer anti-angiogenesis gene therapy is discussed.

ANTI-ANGIOGENESIS CANCER GENE THERPAY STRATEGIES

RNA interference

RNA interference (RNAi) is the sequence-specific gene

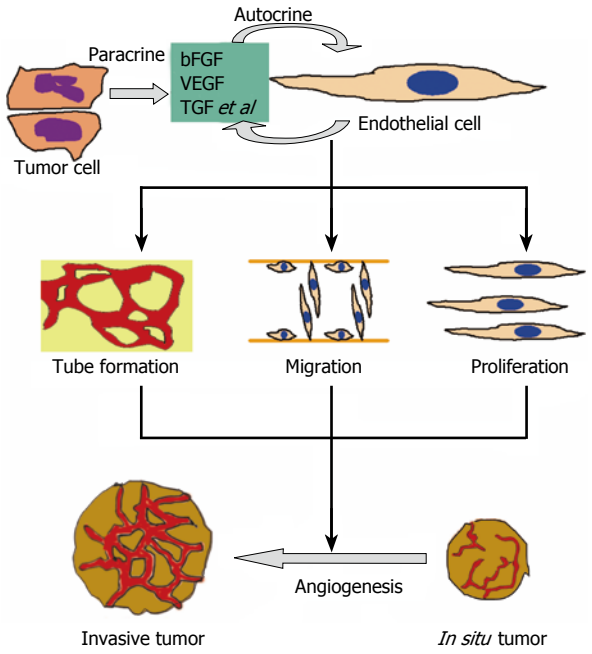

Figure 1 Release of angiogenesis factors mediates the development of invasive tumor.

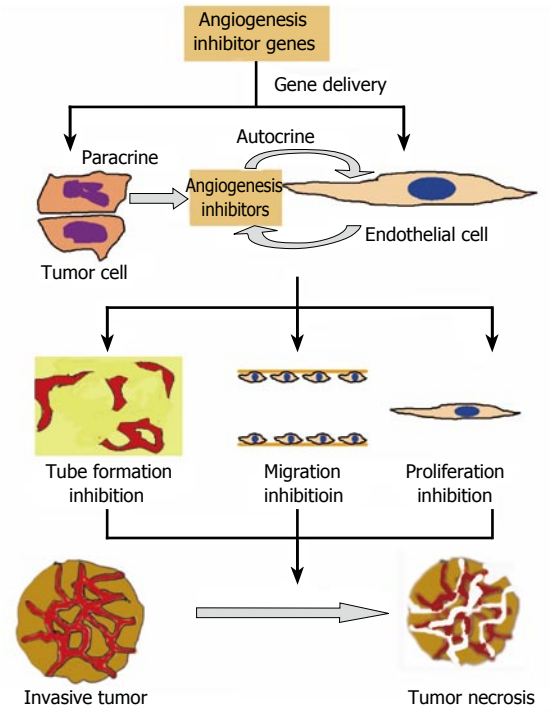

Figure 2 Therapeutic actions of anti-angiogenesis gene therapy.

silencing induced by double-stranded RNA. Introduction of 21-23 small interfering RNAs (siRNAs) of the nucleotide can knock-out the expression of a particular gene[11]. A recent review written by Izquierdo[12] pointed out that siRNA against expression of vascular growth factor receptor (VGFR) can reduce tumor volume by blocking angiogenesis. Kwon and co-workers[13] described a method that can suppress the expression of VGFR-A at both transcriptional and post-transcriptional levels by a combination of zinc finger protein and siRNA. Gondi and co-workers[14] have demonstrated the potential application of RNAi in gene cancer therapy by inhibiting angiogenesis in both *in vivo* and *in vitro* human glioma cell models. Furthermore, it is possible to include more than one antiangiogenesis siRNA into a single retroviral vector because of the small size of siRNA, which could inhibit multiple pathways[12].

Antisense oligodeoxynucleotide

Antisense oligodeoxynucleotide (ODN) is a synthetic molecule that blocks mRNA translation. The blockade of translation of mRNA of pro-angiogenesis factor genes can result in inhibition of tumor growth. Recently, ODN blocking of the expression of VEGF has been shown to be a promising cancer gene therapy. For example, Wang *et al*[15] have successfully reduced VEGF protein expression by 45% in human osteosarcoma cell line by transducing a eukaryotic expression plasmid containing antisense VEGF. Lipiodol is an effective treatment for unresectable liver cancer through transcatheter arterial embolization of the hepatic artery[16]. When VEGF antisense ODN is mixed with lipiodol, this combinational approach is better in inhibiting liver cancer growth, VEGF expression and microvessel density[16].

Expressing the genes of angiogenesis inhibitors

Table 1 summarizes the genes of candidate angiogenesis inhibitors that have been studied recently. In the review published in the *Journal of Translational Medicine*[3], the authors made a thorough account of several candidates. To avoid overlapping of information, we only discuss those candidates that are not covered or recently have demonstrated significant advancement.

Maspin: The Maspin gene is a tumor suppressor gene which is under transcriptional control by p35 and DNA methyltransferase inhibitors. Its gene expression level decreases with malignancy and is lost in metastatic cells[17,18]. Transfection of maspin gene to nude mice could reduce the ability of cells to induce tumors and metastasis[19]. Recently, Watanabe *et al*[18] have shown that adeno-associated virus 2-mediated expression of human maspin can efficiently suppress tumor growth by inhibition of angiogenesis in prostate cancer.

Human ribonuclease inhibitor: Human ribonuclease inhibitor (hRI) is an acidic protein with a molecular weight of 50 kDa. It can inhibit the activity of pancreatic RNase (RNase A)[20]. It is proposed that hRI inhibits angiogenesis by forming a tight complex with its counterpart angiogenin (Ang) which is an angiogenesis factor[21]. Fu *et al*[20] demonstrated that hematopoietic cells carrying the *ri* gene can effectively inhibit tumor growth (by 47%) and reduce tumor microvessel density in mice. They concluded that hRI has the potential utility as a novel antiangiogenesis agent[20].

Survivin: Survivin has been identified as an anti-apoptosis gene over-expressed in cancer and lymphoma[22]. It has been shown that survivin is minimally expressed in endothelium of non-proliferating capillaries of normal skin, whereas it becomes massively up-regulated in newly formed blood vessels of granulation tissue *in vivo*. As a result, manipulation of survivin expression and function in endothelium may influence tumor angiogenesis[23]. Recently, a DNA vaccine targeting survivin and an adeno-associated

viral vector carrying survivin Cys84Ala mutant have been employed to demonstrate the anti-angiogenesis effect on lung tumor and colon cancer cells respectively[24,25].

Soluble FMS-like tyrosine kinase receptor 1: Soluble FMS-like tyrosine kinase receptor 1 (sFlt-1) has been identified as a receptor of vascular endothelial growth factor (VEGF)[26]. It functions by sequestering VEGF and forming inactive heterodimers with other membrane-spanning VEGF receptors both in *in vitro* and *in vivo*[26]. Intramuscular injection of recombinant adeno-associated virus (rAAV) vectors carrying the sFlt-1 gene into nude mice can protect against the human ovarian cancer cell line with increased disease-free survival[27].

Interleukin-12: In 2004 and 2005, Heinzerling *et al*[28] and Imagawa *et al*[29] showed that direct intratumoral injection of interleukin-12 (IL-12) gene produces a reduction in vessel density or angiogenesis in a murine head and neck cancer model and in patients with metastatic melanoma[28,29]. It is worth noting that a few cytokines, like IL-12, have recently been reported to participate in the regulation of the angiogenic switch[30]. These cytokines are related to inflammation. Whether there are relationships between angiogenesis and inflammation may be an interesting topic among scientists in the future.

Pigment epithelium-derived factor: Pigment epithelium-derived factor (PEDF) is a neurotrophic protein and belongs to the serine protease inhibitor (serpin) family[31,32]. It is believed to be a potent inhibitor of angiogenesis[33]. A full-length human PEDF expression vector has been used to transfect the glioma cell line U251, resulting in up-regulation and down-regulation of angiogenesis inhibitors and activators[34].

Tissue inhibitors of metalloproteinase: In 2004, adeno-associated virus-mediated gene transfer of tissue inhibitor of metalloproteinase (TIMP) to animal's tumor also showed that it can inhibit vascular tumor growth and angiogenesis[35].

Angiostatin: Angiostatin is a 38 kD kringle domain of plasminogen and is the most potent and well characterized body's angiogenesis inhibitor[3]. Recently, the effectiveness of co-administration of the mouse angiostatin kringle and the endostatin genes using cationic liposome has been investigated *in vitro* and *in vivo* by Kim *et al*[36].

Melanoma differentiation-associated-7 gene or interleukin-24: Melanoma differentiation-associated-7 (mda-7) gene is a novel melanoma differentiation-associated gene that modulates human melanoma differentiation, growth and progression. It was identified by subtractive hybridization in human melanoma cells by Jiang *et al*[37]. It has been demonstrated that the mda-7 gene functions as a multi-modality anti-cancer agent, possessing both pro-apoptotic and anti-angiogenic properties, and the adenovirus-mediated over-expression of mda-7 gene has the potential therapeutic effects in human lung cancer[38]. More recently, Nishikawa *et al*[39] performed a combination therapy on non-small-cell lung cancer (NSCLC) cell lines and showed that the combination of mda-7 gene therapy and radiotherapy may be a feasible and effective strategy for treatment of NSCLC.

Fragments of hepatocyte growth factor: NK4 is the N-terminal hairpin domain and subsequent four-kringle domains of hepatocyte growth factor (HGF). It was reported that HGF possesses anti-angiogenesis property[40]. A latest trial has been done using hydrodynamics-based gene delivery of naked NK4 plasmid into colon cancer cells in mice. HGF can efficiently express NK4, inhibit liver metastasis and subsequent invasive growth of colon cancer and prolong survival of mice[41]. In addition to NK4, recombinant kringle 1 domain of HGF (HGFK1) has been shown to inhibit bovine aortic endothelial cell proliferation stimulated by basic fibroblast growth factor (bFGF) in a dose-dependent manner[42]. These studies present the potency of the fragments of HGF in inhibiting angiogenesis.

NC1 domains of collagen: Endostatin (from collagen XVIII), restin (from collagen XV), arrestin (α1 chain of collagen IV) and canstatin (α2 chain of collagen IV) are all NC1 domains as reviewed by Tandle *et al*[3]. Recently it has been shown that vastatin, the NCI domain of collagen VIII (α1) possesses anti-angiogenesis ability in bovine aortic endothelial cells[43]. This provides another promising candidate for caner anti-angiogenesis gene therapy.

Table 1 Genes of canadidate angiogenesis inhibitors

Candidate	Reference(s)
16 kD prolactin fragment[1]	3
Angiostatin[1]	3, 36
Arresten[1]	3
Canstatin[1]	3
Endostatin[1]	3
Endothelial-monocyte activating polypeptide-Ⅱ (EMAP-Ⅱ)[1]	3
Fragments of hepatocyte growth factor (HGF)	
NK4	40, 41
HGFK1	42
Human rubonuclease inhibitor (hRI)	20, 21
Interferon-inducible protein-10 (IP-10)[1]	3
Interferons[1]	3
Interleukin-12 (IL-12)[1]	28, 29
Interleukin-18 (IL-18)[1]	3
Interleukin-24 (IL-24)	37, 38, 39
Maspin	17, 18, 19
p53[1]	3
Pigment epithelium-derived factor (PEDF)	31, 32, 33, 34
Platelet factor-4[1]	3
Restin[1]	3
Soluble FMS-like tyrosine kinase receptor 1 (sFlt-1)	26, 27
Survivin	22, 23, 24, 25
Thrombospondin-1 (THBS1)[1]	3
Tissue inhibitors of metalloproteinases (TIMPs)[1]	3, 35
Tumor necrosis factor alpha (TNF-α)[1]	3
Tumstatin[1]	3
Vastatin[1]	43

[1]Candidates that have been covered in Tandle *et al*[3].

GENE DELIVERY SYSTEMS

The viral vectors used for tumor vascular targeting therapy are summarized in a recent review[44]. Tandle *et al*[3] have also discussed some non-viral gene delivery vectors. Again, we will focus on those newly studied viral vectors showing advancement. Table 2 summarizes the vectors that are

Table 2 Gene delivery vectors

Vector	Brief description	Reference (s)
Cationic liposomes	Spherical vesicle made of positively charged lipids, efficient uptake of DNA by the cell	46, 47
Low Voltage Electroporation	Entry of DNA into the cell whose membrane is permeabilized by electric field, efficient gene transfer is ensured	48, 49
Nanoparticles	Submicron-sized particle with the therapeutic agent situated within the matrix or on the surface	3, 45
Measles virus	Contains negative strand RNA molecule, can enter tumor cells without the defensive responses of the tumor	44
Herpes simplex virus	Dobule stranded DNA virus, wide host range, large transgene capacity, long-lasting effect	3, 44
Lentivirus	Derived from HIV, can achieve stable integration of the gene in non-dividing cells	3, 44
Retrovirus	Based on murine leukemia virus, foreign cDNA can be incorporated into host cell genome with high efficiency	3, 44
Replication-competent Retrovirus (RCR)	Modified retroviral vector that can replicate in solid tumor model so the transfer efficiency is enhanced	55, 56
Semliki Forest Virus (SFV)	One type of alphavirus, modification of its RNA genome yield a new expression vector that transfers transgene into tumor	50, 51, 52, 53, 54
Recombinant adenovirus (rAdv)	Double stranded DNA virus, can be produced in high titres and deliver transgene efficiently Special engineered adenovirus: oncolytic and gutless	3, 57, 58, 59, 60, 61, 62, 63, 64
Recombinant adeno-associated virus (rAAV)	Possess the advantages of rAdv and retrovirus, low level of immune response	3, 25, 65, 66, 67, 68

recently used in cancer antiangiogenesis gene therapy.

Nanoparticles: Polymeric drug carriers are used to deliver low molecular mass drugs, oligonucleotides and peptides, which has attracted attention in recent years[3]. Due to their small sizes, nanoparticles penetrate into even small capillaries and are taken up by cells that can deliver targeted drugs to cells or tissues[3]. In 2005, Schiffelers *et al*[45] constructed self-assembling nanoparticles with siRNA as a means to target tumor neovasculature expressing integrins and to deliver siRNA which inhibits VEGF-R2 expression and thereby tumor angiogenesis. They pointed out that this mode of delivery overcomes the pharmacological hurdles of local administration of aqueous siRNA for cancer therapy.

Cationic liposome: The advantages of using a cationic liposome as a vehicle for drug delivery are the enhancement of delivery and expression of the transfected gene. The positive charge significantly increases the uptake of liposome by the endothelial cells of blood vessels in tumor tissues, which has made the cationic liposome useful for delivering tumor targeted drugs[46]. A recent successful case of angiogenesis inhibition using angiostatin and endostatin genes delivered by a cationic liposome has been reported. In addition, modified liposome targeting membrane type-1 matrix metalloproteinase (MT1-MMP) molecules expressed specifically on angiogenesis endothelium and tumor cells, enhances its binding to and accumulates ECs in tumor compared to unmodified liposome[47].

Low voltage electroporation: Electroporation is the formation of pores on the cell surface induced by electric pulse. Direct delivery of plasmid DNA into cells relies on electroporation. *In vivo* electroporation is a novel non-viral means of gene transfer and offers several advantages over viral means such as none of immunogenicity, ease of handling and high gene transfer efficiency[48]. Uesato and co-workers[49] have successfully demonstrated the anti-tumor effect of antiangiogenesis genes, mouse angiostatin and mouse endostatin, delivered to tumors by low-voltage electroporation in 26 models of mouse colon. They have also reported a decrease in microvessel density of tumors[49].

Semliki forest virus: A new expression vector system derived from semiliki forest virus (SFV) was introduced in 1994[50]. This system has been utilized in delivering glycoproteins in a recombinant vaccine study[51]. The vector has also been shown to be a candidate medium for human cancer gene therapy[52]. More recently, SFV vector carrying murine IL-12 gene demonstrated by Doppler ultrasonography, could cause B16 tumor regression through anti-angiogenesis[53]. After this, two IL-12 gene subunits cloned from mouse splenocytes and inserted into an enhanced SFV vector (pSFV10-E) could show complete tumor regression in mice[54].

Replication-competent retroviruses: Retroviruses are a class of virus which has a genome of a single stranded RNA molecule. Vectors derived from murine leukemia virus, a simple retrovirus, have been used in *in vivo* gene transfer in gene therapy. However, the limited efficiency of replication-defective retrovirus vector is a major obstacle in cancer gene therapy[55]. Logg's group in Los Angeles thus developed a replication-competent retrovirus (RCR) vector derived from murine leukemia virus[55]. This vector is able to replicate and transmit a transgene both in culture and in a solid tumor model *in vivo*. By taking advantages from RCR vectors, Sun *et al*[56] transduced RCR vectors carrying the human interferon-inducible protein-10 (IP-10) gene to tumor cells *in vivo* and *in vitro*, showing sustained production of IP-10 in culture and reduced angiogenesis in mice.

Recombinant adenovirus: Adenovirus has a double stranded DNA genome. Recombinant adenovirus (rAdv) vectors containing exogenous genes for *in vivo* transfer derived from adenovirus type 5 are made replication deficient by deletion of the E1 region[57]. rAdv is currently the most widely used gene delivery vector because it

enjoys several advantages like high delivery efficiency into both dividing and non-dividing cells, large ability to package foreign genes, easy to grow to high titers and to be purified, non-oncogenic and high expression of the transgenes[58]. In recent years, phase Ⅰ trials have been undertaken using adenoviral p53 (Adp53) for patients with ovarian cancer[59]. In China, phase Ⅰ and Ⅱ trials using recombinant Adp53 to treat laryngeal cancer (phase Ⅰ), head and neck squamous cell carcinoma (phase Ⅱ) and nasopharyngeal carcinoma (phase Ⅱ) have been undertaken extensively[60-62].

Oncolytic adenovirus is a specially engineered adenovirus which exhibits lytic property of virus replication[63]. This adenoviral system not only offers the advantage of high gene delivery efficiency, but also the ability to select infections of tumor cells[63]. As a result, an amplification effect of the therapeutic gene can be achieved through the lateral spread of the progeny vector[63].

The latest generation of adenoviral vector is the gutless adenovirus. It has become an attractive agent for gene therapy because of the reduction of *in vivo* immune response[64] and long-term sustained expression. However, because of the lack of all viral coding regions, the packaging of this virus requires the presence of helper virus which presents the possibility of contamination[64].

Recombinant adeno-associated virus: Recombinant adeno-associated virus (rAAV) has the advantages of broad host range, low level of immune response, and longevity of gene expression that enable the initiation of a number of clinical trials using this gene delivery system[65]. As reviewed recently, there are 8 well-defined serotypes (serotypes 1-5 and 7-9), and more than 100 variants[66]. The underlying mechanism of the selective tissue tropism of different serotypes remains elusive[66]. For anti-angiogenesis cancer gene therapy using rAAV, recent research examples are focusing on treating colon cancer (*in vitro* and *in vivo*), ovarian cancer (*in vivo*) and human glioblastoma (*in vitro*)[25,67,68].

TUMOR SPECIFICITY AND GENE DELIVERY: LESSONS FROM CLINICAL TRIALS

While previous studies on gene targets are limited to pre-clinical stages, the recombinant proteins of some of these targets have entered clinical trials. Can we learn lessons from the trials to optimize the specificity and efficiency of the candidate gene therapeutics?

Recombinant endostatin is currently the most studied angiogenesis inhibitor in the clinical setting. The earliest phase Ⅰ trials were published in 2002 and 2003[69-72]. However, the results were disappointing. Two very recent reports stated that although the endostatin trials have confirmed the safety of endostatin as a pharmacological agent, it is difficult to establish the biologically effective dose of the recombinant protein[73,74]. To address the problem of effective dose of endostatin, Tjin Tham Sjin *et al*[75] recently demonstrated that adeno-associated viruses carrying canine endostatin can dose-dependently express transgene in the circulation after intramuscular injection in mice. Elevated levels of endostatin remain stable in the circulation for at least 4 mo[75]. Therefore, adeno-associated virus-mediated endostatin gene therapy appears to be a potential therapeutic regime with specific and sustained delivery efficiency.

IL-12 is another widely studied agent with anti-angiogenesis activity in clinical trials. Recombinant human IL-12 protein has entered phase Ⅰ and Ⅱ studies in Germany and United States, respectively[3]. Due to the occurrence of dose-limiting toxicity in some patients, the direction of study has switched to gene therapy approaches[3]. A phase Ⅰ trial involving an adenoviral vector encoding human IL-12 gene has been conducted[76], showing that dose-limited toxicity is significantly increased in tumor infiltration by effector immune cells. Despite the lower anti-tumor power of IL-12 gene therapy in human trials, the concept of stimulation of immune response by specific production of IL-12 inside a tumor is proved[77].

Recently, attention has been paid to combination therapy in which anti-angiogenesis treatment is combined with chemotherapy as well as radiotherapy[78]. Approaches like combination of endostatin and VGFR-2 tyrosine kinase inhibitor and even tri-combination of anti-angiogenesis, chemotherapy and radiotherapy have also been tested[79,80]. Co-targeting of tumor and tumor micro-environment can effectively suppress angiogenesis and tumor growth in the prostate cancer model[81]. A Chinese phase Ⅲ trial using recombinant endostatin in combination with chemotherapy in NSCLC has exhibited a significant increase in response rates and time to progression[73].

Specificity and safety of the vectors are the two main issues that should be addressed in the future. Development of vectors that exhibit superior safety and direct the therapeutic transgene to the right target position of the genome without any random insertion side effects would be a direction for studying human gene therapy against cancer.

CONCLUSION

Targeting angiogenesis is a promising approach in suppressing tumor growth and metastasis. Due to the need for long term administration of the inhibitors, gene therapy has become an alternative which theoretically ensures a sustained availability of the anti-angiogenesis agents. Up till now, researches on anti-angiogenesis cancer gene therapy remain in pre-clinical stage. It is anticipated that when better vectors are developed and the molecular mechanisms of angiogenesis inhibitors against tumor growth are better understood, clinical trials will be undertaken in the future.

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Comparison of gene expression profiles between primary tumor and metastatic lesions in gastric cancer patients using laser microdissection and cDNA microarray

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Abstract

AIM: To study the differential gene expression profiles of target cells in primary gastric cancer and its metastatic lymph nodes using laser microdissection (LMD) in combination with cDNA microarray.

METHODS: Normal gastric tissue samples from 30 healthy individuals, 36 cancer tissue samples from primary gastric carcinoma and lymph node metastasis tissue samples from 58 patients during gastric cancer resection were obtained using LMD in combination with cDNA microarray independently. After P27-based amplification, aRNA from 36 of 58 patients (group 1) with lymph node metastasis and metastatic tissue specimens from the remaining 22 patients (group 2) were applied to cDNA microarray. Semiquantitative reverse transcriptase polymerase chain reaction (RT-PCR) and immunohistochemical assay verified the results of microarray in group 2 and further identified genes differentially expressed in the progression of gastric cancer.

RESULTS: The expression of 10 genes was up-regulated while the expression of 15 genes was down-regulated in 22 gastric carcinoma samples compared with that of genes in the normal controls. The results were confirmed at the level of mRNA and protein, and suggested that four genes (OPCML, RNASE1, YES1 and ACK1) could play a key role in the tumorigenesis and metastasis of gastric cancer. The expression pattern of 3 genes (OPCML, RNASE1 and YES1) was similar to tumor suppressor genes. For example, the expression level of these genes was the highest in normal gastric epithelium, which was decreased in primary carcinoma, and further decreased in metastatic lymph nodes. On the contrary, the expression pattern of gene ACK1 was similar to that of oncogene. Four genes were further identified as differentially expressed genes in the majority of the cases in the progression of gastric cancer.

CONCLUSION: LMD in combination with cDNA microarray provides a unique support foe the identification of early expression profiles of differential genes and the expression pattern of 3 genes (OPCML, RNASE1 and YES1) associated with the progression of gastric cancer. Further study is needed to reveal the molecular mechanism of lymph node metastasis in patients with gastric cancer.

Key words: Gastric cancer; cDNA microarray; Laser microdissection; Reverse transcriptase polymerase chain reaction; P27

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INTRODUCTION

Gastric cancer is one of the leading causes of cancer death in the world, its clinical behavior depends on the potential metastasis of the tumor, and the prognosis of advanced gastric cancers remains very poor. Until now, several molecules have been reported to play an important role in gastrointestinal tumorigenesis and tumor metastasis[1-3], but the molecular mechanisms involved in tumor development and progression remain unclear in gastric cancer[1-3].

In this study, using the combined methods of laser microdissection (LMD), P27-based RNA amplification, and cDNA microarray, we evaluated the differentially expressed genes in primary carcinoma cells and lymph node metastatic cells in 36 of 58 patients. Moreover, we further identified four differentially expressed genes in the remaining 22 patients with progression of gastric cancer by semiquantitative reverse transcriptase polymerase chain reaction (RT-PCR), and the expression patterns of these

four genes were similar to those of tumor suppressor genes or oncogenes.

It has been widely accepted that many malignant tumors contain heterogeneous subpopulations of cells. This heterogeneity exhibits a wide range of genetic, biochemical and immunologic characteristics. It is likely that specific tumor cells or colonies within larger heterogeneous tumor specimens are the forerunners of distant metastases[4]. Therefore, many biologic differences exist in tumor cells of primary carcinoma and metastatic lesions. Furthermore, interaction of tumor cells with their living environment may add more differences to these tumor cells[5]. As a result, tumor metastasis-related genes can be identified by comparing their gene expression profiles.

LMD and cDNA microarray are two of the new emerging techniques in the post-genomic era. LMD is an innovative technique which offers researchers a simple, reliable, rapid and accurate tool for precise and contamination-free procurement of cell groups from tissue sections under direct visualization[6,7].

Large-scale analysis of gene expression with cDNA microarray allows us to evaluate the gene-expression profiles of hundreds to tens of thousands of genes in a single experiment[8]. This techniques is a powerful tool for analyzing the expression of genes which may be correlated with pathological phenotypes of various diseases. However, the expression profile of a specific cell type may be primarily masked or even lost because of the bulky surrounding cells. Therefore, LMD in combination with cDNA microarray can provide a unique opportunity to study gene expression of subpopulations of cells in their native tissue environment *in vivo*.

MATERIALS AND METHODS

Patients with gastric cancer and their pathological samples

Fifty-eight advanced gastric adenocarcinoma (TNM stage Ⅲ-Ⅳ) patients with lymph node metastasis diagnosed by postoperative pathology were investigated in this study. There were 30 male and 28 female patients, whose ages ranged from 45 to 68 years with an average age of 58.7 ± 3.46 years (Table 1). Histologically, 38 patients had moderately differentiated adenocarcinoma and 20 had poorly differentiated adenocarcinoma. All patients underwent gastrectomy with regional lymph nodes dissected and informed consent to participate in this study was obtained from each patient. Tissue blocks of normal gastric epithelium (> 5 cm away from the edge of the tumor), primary tumors and corresponding metastatic lymph nodes were obtained within 30 min after removal from the patient. Each block was cut into 2 pieces, one for routine pathologic diagnosis, and the other for molecular analysis. The latter samples were frozen in liquid nitrogen immediately and stored at -260℃ until use.

Table 1 Characteristics of the studied patients (n = 58)

Characteristics	n (%)
Age (yr)	
Range	48-68
mean ± SD	58.7 ± 3.46
Median	59
Mode	59
Gender	
Male	30 (52)
Female	28 (48)
Tumor location	
Gastric antrum	38 (66)
Gastric body	10 (17)
Gastric cardia	10 (17)
Anemia	
Yes	48 (83)
No	10 (17)
Abdominal pain	
Yes	27 (47)
No	31 (53)
Weight los	
Yes	34 (59)
No	24 (41)
Occult blood test	
Positive	43 (74)
Negative	25 (26)

Laser microdissection and RNA extraction

Before sectioning, tissue blocks were embedded in Tissue Tek OCT compound medium (VWR Scientific Products, San Diego, CA, USA) in a cryostat. Then serial 8-micron thick sections were prepared and mounted onto a foiled slide and stored at -70℃ until use.

Frozen section slides were stained just before laser microdissection on ice. Briefly, the slides were fixed in 70% ethanol for 30 s and stained with 0.1% toluidine blue (TBO) for 15 s, followed by dehydration in 75%, 95% and 100% ethanol respectively for 5 s and dehydration in xylene for 5 min. Once airdried, the sections were laser microdissected with a LMD system (Leica Microsystems, Wetzlar, Germany) and the target cells were selectively collected. Next, total RNA was extracted from the interest cells independently with the RNA-lyase Micro kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. The integrity of the total RNA was measured by Lab-on-chip (Agilent, Palo Alto, USA).

cDNA microarray

We carried out cDNA microarray analysis in 58 metastatic gastric cancer tissue samples of lymph node metastatic gastric cancer, 36 tissue samples of primary gastric cancer and 30 tissue samples of normal gastric epithelium, all technical services were provided by Shanghai Biochip Corporation (Shanghai, China). Two hundred ng of total RNA was amplified for array analysis, 100 ng of total RNA was not amplified for later certification. After P27-based RNA amplification, aliquots (2.5-microgram) of aRNA from primary carcinoma tissues and their corresponding metastatic lymph nodes were labeled with Cy3-dCTP and Cy5-dCTP, respectively. The labeled probes were hybridized with Human cDNA Chip version 2.0 (SBC-R-HC-100-20, Shanghai, China) containing 13 824 genes (including 10 positive controls and 6 negative ones), and the signals were detected by the Agilent scanner (Agilent, Palo Alto, USA). We set the cutoff values for signal intensities, i.e., the signal to noise ratios of Cy3 or Cy5

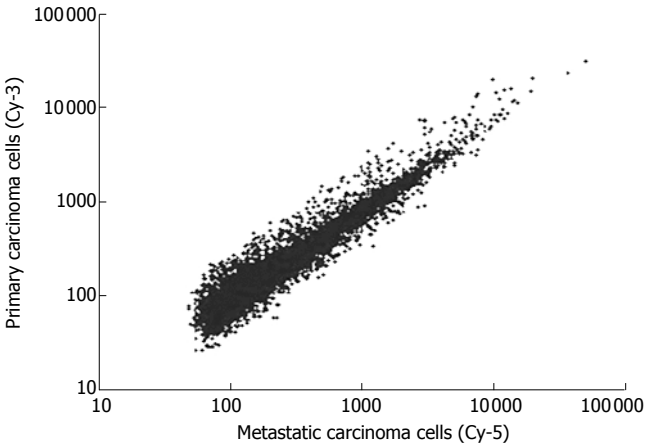

Figure 1 Scatter plots of cDNA microarray analysis. Primary carcinoma cells and lymph node metastatic cells from 58 patients (TNM stage Ⅲ-Ⅳ) with gastric cancer were labeled by Cy-3 and Cy-5 respectively, and hybridized to the cDNA microarray using laser microdissection in combination with microarray.

must be greater than 2. Genes with Cy3:Cy5 ratios > 4 or < 0.25 in the remaining 22 patients were defined as up- or down-regulated genes.

Semiquantitative RT-PCR

Nonamplified total RNA (100 ng) was reverse transcribed into cDNA using Superscript Ⅱ reverse transcriptase (Invitrogen, USA) at 42℃ for 60 min and at 70℃ for 15 min. Each single-stranded cDNA was diluted for subsequent PCR amplification and the content of cDNA was semiquantitatively normalized by housekeeping gene β-actin. PCR conditions for different genes included an initial denaturation at 94℃ for 3 min, followed by 30-35 cycles of denaturation at 94℃ for 30 s, annealing at 94℃ for 30 s, and elongation at 72℃ for 1 min (The primer sequences, annealing temperatures and cycles of each gene are available on request). Amplified PCR products were visualized by electrophoresis on 1% agarose gel containing ethidium bromide.

Immunohistochemical assay

The slides were incubated with rabbit anti-OPCML antibody (BOSTER, USA) at 1:50 dilution. The sections were then stained with KIT SA1028 (BOSTER, USA) according to the manufacturer's instructions. The tissue was counterstained with hematoxylin.

Laser microdissection

Target cells in each sample were successfully laser microdissected. Consequently, about 6×10^6-8×10^6 cells were collected for total RNA extraction, 200-400 ng total RNA was obtained. The integrity of each sample was proven by Lab-on-chip.

Statistical analysis

All data were expressed as mean ± SD. Statistical significance between the two groups was determined by Student's *t*-test using GraphPad Prism version 3.02 for Windows (GraphPad Software Inc., San Diego, USA). $P < 0.05$ was considered statistically significant.

Table 2 Differentially expressed genes detected by cDNA microarray

GeneBank	Description	$C_y3:C_y5$[2]
NM_006500	MCAM (melanoma adhesion molecule)	10.79[1]
NM_002545	OPCML (opioid binding protein/cell adhesion molecule-like)	10.56[1]
NM_002933	RNASE1 (ribonuclease, RNase A family, 1)	6.8[1]
NM_001993	F3 (coagulation factor Ⅲ)	6.29
NM_005433	YES1(v-yes-1 Yamaguchi sarcoma viral oncogene homolog 1	5.916[1]
NM_016525	UBAP (ubiquitin associated protein)	5.876
NM_001428	ENO1 (enolase 1)	5.692[1]
S68616	SLC9A1 (Na+/H+ exchanger NHE-1 isoform)	5.484
NM_003254	TIMP1 (tissue inhibitor of metalloproteinase 1)	5.29[1]
NM_005903	MADH5 (mothers against decapentaplegic, homolog 5)	5.083
U82828	ATM (ataxia telangiectasia)	0.205[1]
NM_006343	MERTK (c-mer proto-oncogene tyrosine kinase)	0.19
NM_002985	SCYA5 (small inducible cytokineA5)	0.19[1]
NM_005348	HSPCA (heat shock 90kD protein 1, alpha)	0.164
NM_003968	UBE1C (ubiquitin-activating enzyme E1C)	0.161
NM_004374	COX6C (cytochrome c oxidase subunit VIc)	0.131
NM_002990	SCYA22 (small inducible cytokine subfamily A, member22)	0.129
NM_005781	ACK1 (activated p21cdc42Hs kinase)	0.128[1]
NM_005139	ANXA3 (annexin A3)	0.124
AF053630	SERPINB1(serine proteinase inhibitor, clade B, member 1)	0.123
NM_012090	MACF1 (microtubule-actin crosslinking factor 1)	0.098
XM_042551	CAMK2A (calcium/calmodulin-dependent protein kinase)	0.0645
NM_000909	NPY1R (neuropeptide Y receptor Y1)	0.0252[1]
NM_015230	CENTD1 (centaurin, delta 1)	0.024
NM_004958	FRAP1 (FK506 binding protein 12-rapamycin associated protein 1)	0.0205

[1]Up-regulated genes were confirmed by semiquantitative RT-PCR in another group of 15 cases; [2]Down-regulated genes and aRNA from primary carcinoma tissues and their corresponding metastatic lymph nodes were labeled with Cy3-dCTP and Cy5-dCTP, respectively.

RESULTS

After P^{27}-based amplification, we evaluated the expression profiles of tumor cells of primary gastric cancer and their corresponding metastatic lymph nodes in 36 patients. Scatter plots of cDNA microarray analysis are shown in Figure 1. Amplified aRNA from primary gastric carcinoma cells (Cy3) and metastatic carcinoma cells (Cy5) were labeled and hybridized to the cDNA microarray.

Analysis of the cDNA microarray data showed that 49 genes (including 31 with unknown function) were over-expressed (Cy3:Cy5 > 4) in primary carcinoma cells from 36 patients. On the other hand, 37 genes (including 9 with unknown function) were significantly suppressed (Cy3:Cy5 < 0.25) in primary carcinoma cells from 36 patients. The up-regulated genes were related to cell adhesion, cytoskeleton, cell defense and metabolism. Meanwhile, the down-regulated genes included those associated with cell development, cell cycle, signal transduction, adhesion, cell

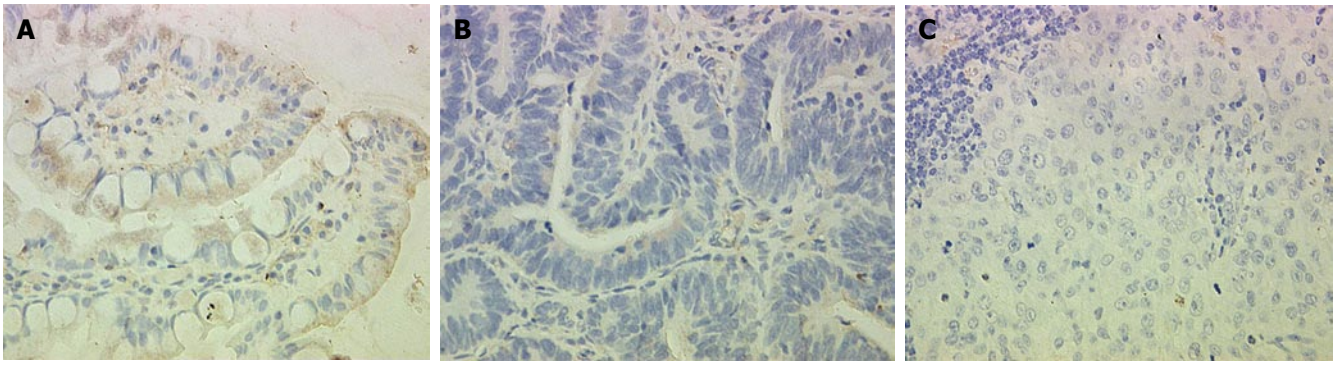

Figure 2 Immunohistochemical staining of OPCML. **A**: Normal gastric epithelium showing abundant and strong immunoreactivity; **B**: Primary gastric cancer demonstrating weak immunoreactivity; **C**: lymph node metastastasis showing rare immunoreactivity (× 400).

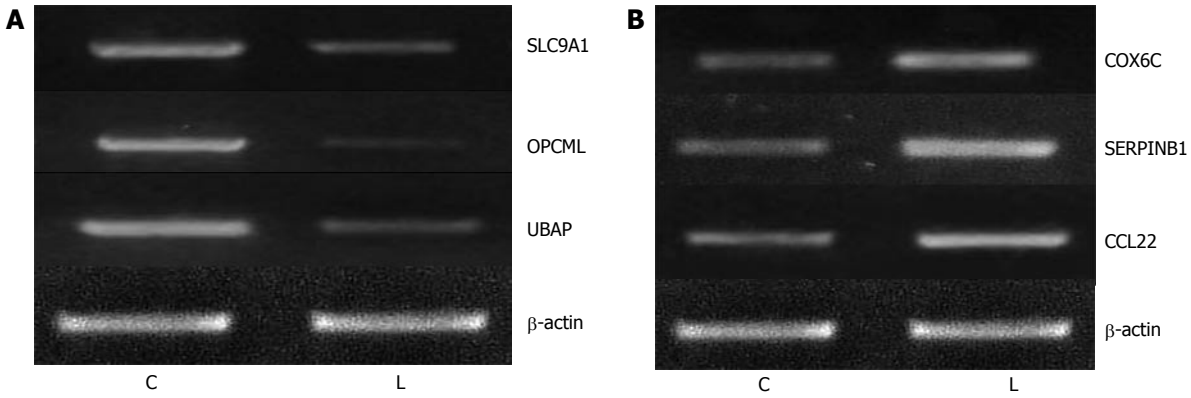

Figure 3 Validation of microarray data by semiquantitative RT-PCR. **A**: Expression levels of 3 genes (SLC9A1, OPCML and UBAP) was up-regulated in primary carcinoma; **B**: Expression of 3 genes (COX6C, SERPINB1 and CCL22) was down-regulated in primary carcinoma. C: A sample from the primary carcinoma cells; L: A sample from the metastatic gastric carcinoma cells.

defense, gene expression and cell metabolism (Table 2).

Confirmation of microarray findings

To examine the reliability of microarray data, we confirmed our data at the level of mRNA and protein. First, we selected 3 up-regulated genes (SLC9A1, OPCML and UBAP) and 3 down-regulated genes (COX6C, SERPINB1 and CCL22) to measure their expression levels by semiquantitative RT-PCR. To obtain truly comparable results, we used the unamplified total RNA (from the same batch used for array hybridizations) as the template. The results were very similar to the microarray data on these genes.

To confirm our data at the protein level, we performed immunohistochemical analysis of OPCML. The results were in parallel with the differential expression pattern detected by cDNA microarray and RT-PCR (Figure 2).

Overall, the above results demonstrated that the samples obtained by P27-based amplification well reflected the status of the original RNA in a proportional manner, and supported the reliability of our expression data (Figure 3).

Expression of differential genes in progression of gastric cancer

By semiquantitative RT-PCR, we further evaluated the expression levels of 25 selected genes, including 10 up-regulated and 15 down-regulated genes, in microdissected normal gastric epithelial cells from 30 healthy individuals, primary gastric cancer cells from 36 patients and lymph node metastatic cells from the remaining 22 patients. First, we investigated the expression levels of these genes in primary gastric cancer and lymph node metastatic cells. The results showed that the expression of 12 genes had the same pattern in > 50% (12/22) patients as revealed by cDNA microarray.

We further measured the expression levels of these 12 genes in paired normal gastric epithelium and primary carcinoma samples from the same 22 patients. We found that the expression pattern of 3 genes (OPCML, RNASE1 and YES1) was similar to that of tumor suppressor genes in > 50% (8/15) patients. For example, the expression level of these genes was the highest in normal gastric epithelium, which was decreased in primary carcinoma and further decreased compared to that in primary gastric cancer patients with lymph node metastasis and normal controls ($P < 0.05$). Meanwhile, the expression level of ACK1 demonstrated the opposite tendency, the pattern of which was similar to that of oncogene in > 50% (12/22) patients (Figure 4). The expression pattern of OPCML was also confirmed at the protein level by immunohistochemical staining (Figure 2).

DISCUSSION

cDNA microarrays allow an effective investigation of functional genomics. However, the existence of bulky surrounding cells produces much useless noise information because of its high sensitivity[9,10]. Therefore, selection of cancer cells using LMD is of indispensable value in combination with the cDNA microarray. The LMD system used in this study integrates a UV laser of 337 nm wavelength with an upright microscope. The ultraviolet laser microbeam causes dissection by local photolysis of the supporter membrane and tissue section due to the high photon density of the microbeam rather than by local heating or coagulation. The cut sample falls down into PCR tubes placed underneath by gravity without any mechanical contact or further destroying energy and the integrity of the extracted mRNA is maximally kept.

Metastasis of cancer is a highly selective sequential step which favors the survival of a subpopulation of metastatic cells preexisting within the primary tumor mass to produce clinically relevant metastases. The metastatic cells exhibit a complex phenotype that is regulated by transient or permanent changes in different genes at the DNA and/or mRNA level(s). This has also been proved in gastric cancer by other researchers[8,9,11].

Differential expression profiles of laser microdissected primary gastric cancer cells and lymph node metastatic cells were discovered using cDNA microarray consisting of 13824 genes, demonstrating that analysis of gene-expression profiles can be performed using LMD, P27-based RNA amplification and cDNA microarray[1,11]. Although the majority of these genes have been implicated in various aspects of tumor biology, few are associated with gastric cancer.

Among the above genes, some may be differentially expressed because of different living environments[5,12-17]. Therefore, to further confirm and screen the results of cDNA microarray, we measured the expression levels of 25 selected genes in 22 patients by semiquantitative RT-PCR. These target cells were collected by LMD, and the normal gastric epithelium was included. As a result, we identified 4 genes, the expression level of which was different not only between primary carcinoma and metastatic lymph nodes (the same results as cDNA microarray), but also between normal gastric epithelium and primary tumor, suggesting that these four genes play a key role in the tumorigenesis and metastasis of gastric cancer. The expression pattern of 3 genes (OPCML, RNASE1 and YES1) is similar to that of tumor suppressor genes. For example, the expression of these genes is the highest in normal gastric epithelium, which is decreased in primary carcinoma, and further decreased in metastatic lymph nodes. OPCML encodes a member of the IgLON subfamily in the immunoglobulin protein superfamily and acts as a GPI-anchored protein. It was reported that OPCML has tumor-suppressor function in epithelial ovarian cancer[11,18-20], which is in accordance with our findings in gastric cancer. Interestingly, YES1 is the cellular homolog of a virus oncogene associated with esophageal tumorigenesis[12,21-24], which is contrary to our results. The RNAS gene encodes a member of the pancreatic-type of secretory ribonuclease, a subset of the ribonuclease A superfamily, and has no relationship with human cancers.

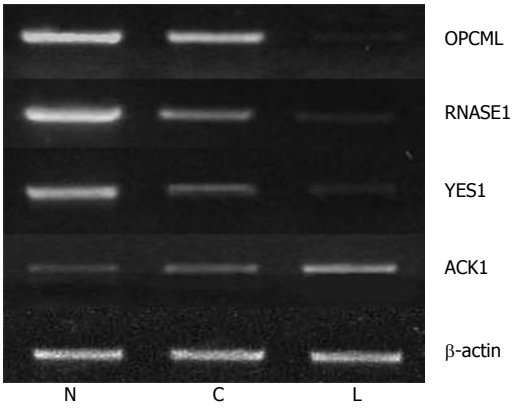

Figure 4 Identification of differentially expressed genes in progrssive gastric cancer by semiquantitative RT-PCR. N: A sample from normal gastric epithelium; C: Signifies a sample from the primary carcinoma cells; and L: A sample from the metastatic carcinoma cells.

In this study, the expression pattern of gene ACK1 was different from that of the above mentioned genes, but similar to that of oncogenes. ACK1 encodes a tyrosine kinase that binds to Cdc42Hs in its GTP-bound form and inhibits intrinsic and GTPase-activating protein (GAP)-stimulated GTPase activity of Cdc42Hs. It is directly linked to a tyrosine phosphorylation signal transduction pathway, but its effect on tumor progression has not been reported[12-17].

Both tumor suppressor gene and oncogene are important target molecules in clinical diagnosis and treatment of malignant gastric tumors. The different expressions of these four genes have not been reported during the progression of gastric cancer.

In conclusion, analysis of gene expression profiles by LMD, P27-based amplification, and cDNA microarray can provide useful information for clarifying the mechanism underlying the development and metastasis of gastric cancer[25-29], not only revealing the differentially expressed genes in progression of gastric cancer, but also providing information for identifying novel diagnostic and therapeutic targets.

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Efficacy and safety of thalidomide in patients with hepatocellular carcinoma

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Abstract

AIM: To evaluate which patients with hepatocellular carcinoma (HCC) are most likely to respond to thalidomide treatment.

METHODS: From July 2002 to July 2004, patients with HCC who received thalidomide treatment, were enrolled. We extracted relevant data from the patients' medical records, including history and type of hepatitis, comorbidity, serum α-fetoprotein (α-FP) level, volumetric changes in tumor, length of survival, and the dose, duration, side effects of thalidomide treatment. The tumor response was evaluated. On the basis of these data, the patients were divided into two groups: those with either partial response or stable disease (PR + SD group) and those with progressive disease (PD group).

RESULTS: Two of 42 (5%) patients had a partial tumor response after treatment with thalidomide, 200 mg/d, and 9 (21%) had stable disease. Patients in the PR + SD group all had cirrhosis. Comparing patients with and without cirrhosis, the former were more likely to respond to thalidomide therapy (PR + SD: 100% *vs* PD: 64.5%, $P = 0.041 < 0.05$). Thalidomide was significantly more likely to be effective in tumors smaller than 5 cm (PR + SD: 63.6% *vs* PD: 25.8%, $P = 0.034 < 0.05$). Compared with patients with progressive disease (PD), patients in the PR + SD group had a higher total dose of thalidomide (13669.4 ± 8446.0 mg *vs* 22022.7 ± 11461.4 mg, $P = 0.023 < 0.05$) and a longer survival (181.0 ± 107.1 d *vs* 304.4 ± 167.1 d, $P = 0.047 < 0.05$). Patients with comorbid disease had a significantly greater incidence of adverse reactions than those without (93.8% *vs* 60.0%, $P = 0.021 < 0.05$). The average number of adverse reactions in each person with a comorbid condition was twice as high as in those without other diseases (2.2 ± 1.3 *vs* 1.1 ± 1.2; $P = 0.022 < 0.05$).

CONCLUSION: Thalidomide therapy is most likely to be effective in patients with early stage small HCC, especially in those with other underlying diseases. A low dose (200 mg/d) of thalidomide is recommended to continue the treatment long enough to make it more effective.

Key words: Thalidomide; Hepatocellular carcinoma; Antiangiogenic agents; Adverse events

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most prevalent cancer in the world. About one million people die of it each year[1,2]. Particularly in Taiwan, it poses a tremendous threat to citizens' health and lives. HCC has been the leading cause of cancer death in Taiwan for the past two years, leading to about 6000 to 7000 deaths each year.

Potentially curative therapies for HCC include surgical resection, liver transplantation, and percutaneous ablation. However, HCC is often diagnosed at an advanced stage when curative therapy is likely to be of little value. Thus, most patients are candidates for palliative treatment, which includes transarterial embolization (with or without chemotherapy), hormonal therapy, interferon therapy, *etc.* Transarterial embolization has been shown to improve survival[1]. The response rate to systemic chemotherapy is generally less than 30%[1], and patients often tolerate it poorly. A further difficulty is that about 80% of patients with HCC also have cirrhosis, which severely limits the utility of both surgery and chemotherapy. Therefore, we urgently need new therapeutic approaches for HCC.

In 1971, Dr. Judah Folkman suggested using anti-angiogenic agents to inhibit tumors, a proposal which has made a new form of anticancer treatment possible. Cancer cells whose blood supply is disrupted are starved of nutrients and oxygen, leading to atrophy and death. Endothelial cells play a key role in angiogenesis, forming the external theca of new blood vessels and possessing a strong ability to reproduce and migrate. Proteins known to activate endothelial cells include vascular endothelial growth factor (VEGF) and basic fibroblast growth factor

(BFGF), which are regarded as important contributors to the growth of new blood vessels. HCC is a hypervascular tumor, with high concentrations of VEGF and BFGF in tumor cells and blood[3,4]. It is therefore reasonable to infer that anti-angiogenesis may inhibit the growth of HCC.

A number of anti-angiogenic agents have been developed, some of which are in phase Ⅱ or Ⅲ clinical trials. Thalidomide is such a drug. It was widely used in the 1950s as a sedative and anti-emetic agent but was found to be teratogenic. Its use has been therefore prohibited in Europe and America. However, in recent years, thalidomide has been found to have many pharmacological properties, including inhibition of angiogenesis and inflammation and regulation of immunity. Thalidomide apparently blocks factors such as VEGF and BFGF of cancer cells[5,6], thereby inhibiting angiogenesis. In some cancers strongly dependent on angiogenesis, such as Kaposi's sarcoma and metastatic prostate cancer, thalidomide has been used with some success[7,8]. HCC, also strongly dependent on angiogenesis, is treated with thalidomide in Taiwan.

As with any newly proposed therapy, it is important to identify those patients most likely to benefit from the treatment while minimizing the risk of adverse effects. This understanding may also facilitate conservation of medical resources when the drug is unlikely to help. We designed this study to evaluate the efficacy and adverse effects of thalidomide in HCC by retrospectively reviewing the results in a series of patients in order to identify those most likely to benefit from the treatment while avoiding serious adverse effects.

MATERIALS AND METHODS

Patients

From July 2002 to July 2004, 159 patients with HCC were treated with thalidomide. All the patients were informed of the necessity of contraception and the benefits and risks of thalidomide treatment. They gave their informed consent to receive it by the Medicine Advisory Committee of Mackay Memorial Medical Center and the Department of Public Health in Taiwan. HCC was diagnosed by histologic examination, imaging findings (abdominal sonography and computed tomography), or a serum α-fetoprotein (α-FP) level greater than 400 ng/mL. The disease was staged by the pathologic tumor-node-metastasis (pTNM) system, the Okuda system for HCC, and the Cancer of the Liver Italian Program (CLIP) scoring system[9-11]. We extracted relevant data from the patients' medical records, including age, gender, history and type of hepatitis, comorbidity, duration of HCC, serum α-FP level, volumetric changes in tumor, length of survival, and the dose, duration, side effects of thalidomide treatment. Patients were excluded from analysis if they took thalidomide for less than one month, had other therapy after thalidomide, or if the data were inadequate in the record to define the stage or treatment response.

Evaluation of tumor response

The tumor response was evaluated according to the imaging findings one month before and after administration of thalidomide according to the new version of the Response Evaluation Criteria in Solid Tumor (RECIST) Guidelines[12], as well as α-FP levels before and after thalidomide treatment. On the basis of these data, the patients were divided into two groups: those with either partial response or stable disease (PR + SD group) and those with progressive disease (PD group).

Thalidomide therapy

Thalidomide (50 mg tablet marketed as Thado®, Taiwan Tung Yang Biopharm Co. Ltd., Taipei) in a dose of 50 to 200 mg/d, was give twice daily in the morning and evening. The dose was adjusted according to the clinical response and side effects. Thalidomide-related toxicity was classified by the Common Terminology Criteria for Adverse Events v 3.0 (CTCAE) established by the National Cancer Institute (NCI)[13].

Statistical analysis

The clinical characteristics, dose of thalidomide, treatment duration, and length of survival of the two groups were compared by using the Mann Whitney *U* test and Fisher's exact test. The relation between comorbidity and adverse reactions was examined by Fisher's exact test. $P < 0.05$ was considered statistically significant. The overall survival was calculated by the Kaplan-Meier method from the time of the first dose of thalidomide to the patient's death or the last follow-up. The statistical software package used was SPSS (version 10).

RESULTS

Patients

Of the 159 patients with HCC treated with thalidomide, 57 took thalidomide for less than one month, and 35 were given other treatment in addition to thalidomide. The data for 25 patients were incomplete, leaving records for 42 patients that were available for analysis. The clinical characteristics are shown in Table 1. Thirty-one (74%) patients were older than 60 years. Cirrhosis was present in 31, and 32 (76%) had comorbid conditions. The prevalence of hepatitis B (15/42) and C (11/42) was similar. Twenty-one (50%) of the patients had TNM stage ⅢA, and the majority had their liver function classified as Child-Pugh A or Okuda Ⅱ. The most frequent CLIP score was 3 (15/42).

Tumor Response

Of the 42 patients, 2 had a partial tumor response to thalidomide at a dose of 200mg/day and a decrease in α-FP level. They both had TNM stage ⅢA disease, Okuda phase Ⅰ or Ⅱ, and a CLIP score of 3 or 4. Another 9 patients had stable disease, 4 of whom also had a fall in serum α-FP level. The remaining 31 patients had disease that continued to progress after thalidomide. These results were comparable to those of other studies[14-19] (Table 2), none of which showed an objective tumor response rate above 10%. The mean time to partial response in our patients was 34 d. The median survival of all 42 patients was 319 d (range, 144 to 494 d), the one-year survival was 14%. The median survival of the PR + SD group was 524 d (range, 218 to 830 d) (Figure 1).

Table 1 Clinical characteristics of 42 patients with HCC

Characteristic	*n*	yr	%
Age			
Median		67	
Range		32-84	
Sex			
Male	11		26
Female	31		74
Comorbidity			
Absent	10		24
Present	32		76
Cirrhosis			
Absent	11		26
Present	31		74
Type of hepatitis			
Hepatitis B	15		36
Hepatitis C	11		26
Hepatitis B + hepatitis C	1		2
Hepatitis B + hepatitis D	1		2
No record	14		33
TNM stage			
Ⅰ	2		5
Ⅱ	6		14
ⅢA	21		50
ⅢB	2		5
ⅣA	7		17
ⅣB	4		10
Child-Pugh classification			
Grade A	25		60
Grade B	14		33
Grade C	3		7
Okuda stage			
Ⅰ	11		26
Ⅱ	27		64
Ⅲ	4		10
CLIP score			
0	2		5
1	10		24
2	7		17
3	15		36
4	4		10
5	4		10
6	0		0
α-fetoprotein			
> 400 ng/mL	21		50
⩽ 400 ng/mL	21		50
Pre thalidomide therapy			
TAE[1]	26		62
PEI + TAE[1]	3		7
Radiation + TAE[1]	2		5
Surgery + PEI[2]	1		2
Chemotherapy	1		2
No therapy	9		21

[1]Transarterial embolization; [2]Percutaneous ethanol injection.

Table 2 Response rates of HCC to thalidomide

Author	*n*	Tumor response, *n* (%)				
		CR[1]	PR[2]	SD[3]	PD[4]	CR[1] + PR[2] + SD[3]
Kong *et al*[14]	11	0	1 (9)	4 (36)	6 (55)	5 (46)
Lin *at al*[15]	27	0	1 (4)	1 (4)	25 (93)	2 (7)
Feun *et al*[16]	7	0	0	0	7 (100)	0
Schwartz *et al*[17]	30	1 (3)	1 (3)	9 (30)	19 (63)	11 (37)
Hsu *et al*[18]	63	1 (2)	3 (5)	20 (32)	39 (62)	24 (38)
Wang *et al*[19]	99	0	6 (6)	-	- -	
Chiou *et al*	42	0	2 (5)	9 (21)	31 (74)	11 (26)

[1]Complete response; [2]Partial response; [3]Stable disease; [4]Progressive disease.

Table 3 Comparison of patients with partial response or stable disease with patients with progressive disease

Characteristic	PR[1] + SD[2] *n*	PD[3] *n*	*P*
Sex			1.0
Male	8	23	
Female	3	8	
Age			0.234
> 60 yr	10	21	
⩽ 60 yr	1	10	
Cirrhosis			0.041[a]
Absent	0	11	
Present	11	20	
Hepatitis			
Hepatitis B	4	13	1.0
Hepatitis C	3	9	1.0
TNM stage			
Ⅰ + Ⅱ	3	4	1.0
ⅢA + ⅢB	4	19	0.180
ⅣA + ⅣB	4	8	1.0
Child-Pugh classification			
Grade A	5	21	0.280
Grade B	6	8	0.136
Grade C	0	2	1.0
Okuda stage			
Ⅰ	3	8	1.0
Ⅱ	8	19	0.717
Ⅲ	0	4	0.558
CLIP score			
0-2	7	13	0.298
3-6	4	18	0.298
α-fetoprotein			0.081
> 400 ng/mL	3	19	
< 400 ng/mL	8	12	
Tumor size			0.034[a]
⩽ 5 cm	7	8	
> 5 cm	4	23	

[1]Partial response; [2]Stable disease; [3]Progressive disease; [a]$P < 0.05$ between the two groups.

Table 3 shows that all patients in the PR + SD group had cirrhosis, suggesting that cirrhosis might influence the efficacy of thalidomide. Patients in this group were more likely to have a CLIP score of 0 to 2 (7/11) or an α-FP level of < 400 ng/mL (8/11). Response to thalidomide was significantly more likely when the tumor was < 5 cm (PR + SD: 63.6% *vs* PD: 25.8%, $P = 0.034 < 0.05$). Other clinical characteristics did not differ significantly between the groups.

The duration of thalidomide therapy was longer in the PR + SD group (Table 4). Patients in the PR + SD group had a significantly higher total dose and a significantly longer survival than those in the PD group.

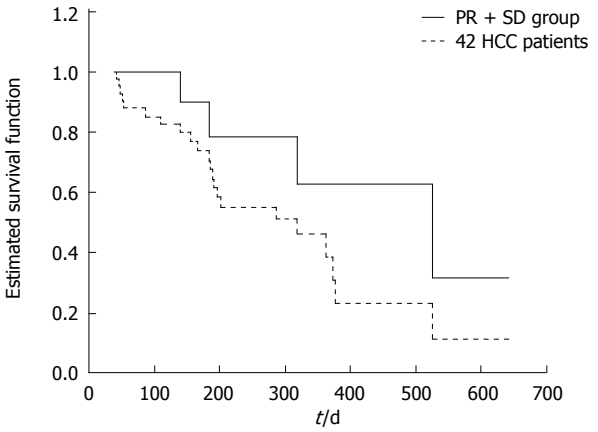

Figure 1 Survival curves in patients with HCC on thalidomide.

Table 4 Comparison of thalidomide treatment and survival between the two groups

	PR[1] + SD[2]	PD[3]	*P*
Duration (d)	167.1 ± 98.9	107.7 ± 71.7	0.069
Total dose (mg)	22022.7 ± 11461.4	13669.4 ± 8446.0	0.023[a]
Survival (d)	304.4 ± 167.1	181.0 ± 107.1	0.047[c]

[1]Partial response; [2]Stable disease; [3]Progressive disease; [a]$P < 0.05$, [c]$P < 0.05$ *vs* duration, between the two groups.

Table 5 Thalidomide-related adverse events in patients with HCC

	Grade of severity (*n*)					Total
Adverse event	G[1]	G[2]	G[3]	G[4]	G[5]	*n* (%)
Constipation	2	22	0	0	0	24 (57)
Skin rash	6	8	3	0	0	17 (41)
Low leg edema	1	10	0	0	0	11 (26)
Dizziness	3	5	1	0	0	9 (21)
Lethargy	3	2	2	0	0	7 (17)
Nausea/vomiting	1	5	0	0	0	6 (14)
Neurosensory	3	0	0	0	0	3 (7)
Dyspnea	0	2	0	0	0	2 (5)
Leukopenia	1	0	0	0	0	1 (2)
Thrombocytopenic purpura	0	1	0	0	0	1 (2)
Insomnia	0	1	0	0	0	1 (2)
Alopecia	1	0	0	0	0	1 (2)

[1]mild adverse event; [2]Moderate adverse event; [3]Severe adverse event; [4]Life-threatening or disabling adverse event; [5]Death related to adverse event.

Thalidomide therapy

Twenty patients began their treatment with a dose of 200 mg/d and the remaining 22100-150 mg/d initially. Ten patients had their dose escalated to 200 mg according to treatment response, while three patients had their dose reduced because of thalidomide- induced drowsiness, dizziness and constipation, and 2 discontinued it entirely. Most adverse reactions were slight or moderate, only 6 (14%) events (skin rash in 3, dizziness in 1, and lethargy in 2) were severe. The most common adverse reactions were constipation and skin rash, with an incidence of 57% and 41%, respectively (Table 5).

Table 6 Thalidomide-related adverse events and comorbidity

Adverse drug events per patient	Comorbidity (*n*)	
	Absent	Present
1	3	10
2	1	7
3	2	6
4	0	7

Table 7 Correlation between thalidomide-related adverse events and comorbidity

	Comorbidity (*n*)		*P*
	Absent	Present	
Without adverse events	4	2	0.021
With adverse drug events[a]	6	30	
Adverse events per patient[c]	1.1 ± 1.2	2.2 ± 1.3	0.022

[a]$P < 0.05$, [c]$P < 0.05$ *vs* without adverse events, between groups of with comorbidity and without comorbidity.

Tables 6 and 7 show the relationship between thalidomide-induced adverse reactions and comorbidity. Adverse reactions were more likely to occur in patients with an other underlying diseases (93.8% *vs* 60.0%, $P = 0.021 < 0.05$). The average number of adverse reactions in each person with a comorbid condition was twice as high as in those without other diseases (2.2 ± 1.3 *vs* 1.1 ± 1.2, $P = 0.022 < 0.05$).

DISCUSSION

In our series, the partial response rate of HCC to thalidomide at 100 to 200 mg/d was 5%, 21% of patients who had stabilization of their disease after treatment with thalidomide. These results generally agree with those of other researchers. In our study, two thirds of the patients were older than 60 and had cirrhosis or other comorbid conditions. Most of them had TNM stage ⅢA disease or were in Okuda class Ⅱ.

The pharmacological mechanisms of thalidomide include anti-angiogenic, immunomodulatory and anti-inflammatory effects. The drug inhibits angiogenesis in tumors by blocking the activity of VEGF and BFGF[20]. The immunomodulatory effects include induction of TNF-α mRNA degradation, thereby inhibiting the synthesis of TNF-α[21]. It also downregulates lymphocyte surface molecules, lowering the CD4:CD8 peripheral lymphocyte ratio. It can inhibit chemotaxis of neutrophils, interleukin-6 and interleukin-12, and stimulate the synthesis of interleukin-2[22]. In addition, it inhibits the activity of IκB kinase, thus blocking the activity of nuclear factor-κB[23].

Because of its many pharmacological actions, thalidomide is now widely used in the treatment of a number of diseases. In 1999, it was approved by the FDA to treat erythema nodosum leprosum. It has been used for aphthous ulcers in HIV-positive patients, graft versus

host disease after bone marrow grafting, tuberculosis, sarcoidosis, inflammatory bowel disease, rheumatoid arthritis, as well as for some cancers such as multiple myeloma, renal cell carcinoma, brain tumors, prostate cancer, melanoma and Kaposi sarcoma[19,23]. The response of multiple myelomas to thalidomide (600 to 800 mg daily) is about 30%, and increases to 60% when combined with dexamethasone. The therapeutic effect of thalidomide in HCC is thus not as satisfactory as in multiple myeloma. The reported response is 40% in Kaposi sarcoma and 15%-27% in prostate cancer[23].

In our study, the plasma concentrations of VEGF and BFGF in multiple myeloma remained unchanged irrespective of whether the disease responded to thalidomide. It is likely that there are mechanisms other than the inhibition of angiogenesis that are responsible for the drug's efficacy in multiple myeloma. For example, there are decreased adhesion of multiple myeloma cells to stromal cells, reduced secretion of cytokines, and activation of caspase-8, leading to death of multiple myeloma cells[24].

Because of the different structures and functions of blood vessels in different tissues and tumors at different sites and the genetic instability and biologic heterogeneity of tumor cells, new blood vessels in tumors may be quite diverse. There are therefore considerable complexity and unpredictability of tumor microvasculature[25]. This may help explain why thalidomide has different effects on different malignancies. The relatively poor response of HCC compared to multiple myeloma may be related to the differences in angiogenesis.

In our series, only about one fifth of the patients had their disease stabilized after thalidomide therapy, indicating that thalidomide helps only a few patients with advanced HCC, and rarely results in a decrease in tumor size. The median survival in the PR + SD group was 524 d (range, 218-830 d) compared with 319 d (range, 144-494 d) of all 42 patients. The one-year survival was only 14%, suggesting that thalidomide is not very effective in prolonging the life of HCC patients.

Our results did show that patients with cirrhosis tolerated thalidomide well and even had a somewhat better response to treatment. The reason why response in those with tumors smaller than 5 cm is better may be that thalidomide can prominently inhibit formation of new blood vessels rather than disrupt existing mature vessels. Larger tumors with mature vasculature may therefore be relatively resistant to the action of thalidomide. The maximum therapeutic effect may thus be seen in smaller tumors with newer vessels.

In the PR + SD group 64% of patients had a CLIP score of 0 to 2, compared with 42% in the PD group. Similarly more patients in the PR + SD group (73%) had an α-FP level of < 400 ng/mL compared with the PD group (39%). The PR + SD group also took a significantly higher total dose of thalidomide (22022.7 ± 11461.4 mg *vs* 13 669.4 ± 8446.0 mg, $P = 0.023$) and had a significantly longer survival (304.4 ± 167.1 d *vs* 181.0 ± 107.1 d; $P = 0.047$). These results indicate that treatment of earlier stage HCC with thalidomide may leave enough time for the drug to produce a significant therapeutic effect.

A commonly-reported adverse effect in patients treated with thalidomide for multiple myeloma is venous thromboembolism, particularly when combined with doxorubicin or with dexamethasone[27]. None of the patients in our series had thromboembolism, perhaps because we used smaller doses than the commonly-used doses for multiple myeloma. The most common adverse reactions in our series were constipation (57%) and skin rash (41%). Severe reactions were uncommon (14%), and only 5 patients required a dose adjustment or discontinuation of treatment. Three patients developed slight peripheral neuropathy, and one of them had a dose adjustment from 200 mg to 100 mg. Thalidomide-related peripheral neuropathy may occur in patients on long-term treatment and is irreversible. Therefore, if it occurs, the drug should be stopped or the dose is reduced. Generally, the adverse reactions caused by thalidomide are dose- and duration-related[27]. We also found a significant positive correlation between adverse reactions and comorbidity ($P = 0.021$) of diabetes mellitus, hypertension, ischemic heart disease, peptic ulcer, chronic obstructive lung disease and benign prostatic hyperplasia. In considering therapy for patients with HCC, the higher risk of adverse reactions to thalidomide in the presence of other diseases should be taken into account.

Our study is limited by the fact that it was retrospective. It would be difficult to collect enough patients with HCC in similar stages for comparison in a prospective study. We also were only able to analyze the data from a relatively small number of patients, which might skew the results to some extent. However, thalidomide is unlikely to be effective in a large proportion of patients with HCC. This makes it even more important to identify those who are likely to benefit from thalidomide treatment.

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Proposal of a new and simple staging system of colorectal liver metastasis

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Abstract

AIM: To create a new, simple and useful staging system for colorectal liver metastasis analogous to the Tumor Node Metastasis classification system of International Union Against Cancer.

METHODS: A retrospective review was undertaken of 81 consecutive patients who underwent partial hepatectomy for colorectal liver metastases (group 1). Clinical and pathological features of both primary and metastatic liver cancers were entered into a multivariate analysis to determine independent variables helpful in accurately predicting long-term prognosis after hepatectomy. Using selected variables, we created a new staging system like TNM classification. The usefulness of the new staging system was examined in a series of 92 patients from another hospital (group 2).

RESULTS: Multivariate analysis showed that 81 patients in group 1 had significant multiple hepatic tumors with the largest tumor being more than 5 cm in diameter, resectable extrahepatic distant metastases, and independent prognostic factors for poor survival after hepatectomy. Using these three variables, we created a new staging system to classify patients with colorectal liver metastases. Finally, our new staging system classified the patients both in group 1 and in group 2.

CONCLUSION: Our new staging system of colorectal liver metastasis is simple and useful for staging patients.

Key words: Colorectal liver metastasis; Staging system; Prognostic factor

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INTRODUCTION

It is well accepted that hepatic resection of colorectal liver metastases is a beneficial clinical procedure in that it reportedly improves 5-year survival rates of affected patients by as high as 30%-50% following curative hepatic resection[1-14]. Although this procedure is performed worldwide by a number of liver surgeons, it is often hazardous to compare results from different studies due to the fact that there is no universally accepted classification system for staging colorectal hepatic metastatic diseases. Several recent papers have offered classification systems based on a variety of variables of colorectal liver metastasis[1-6], including the number of metastatic nodules[1,4-6], the size of metastases[4,6], the sites of unilobar or bilobar involvement[1,4], the extent of liver involvement ($\leqslant$ 25%, and $\leqslant$ 50%)[1,3], the chronology of synchronous or metachronous disease[1,5,6], the invasion to major vessels or bile ducts[2], the presence of extrahepatic metastasis[2-4], the performance status and serum alkaline phosphatase[3]. However, it is uncertain whether these classification systems are accepted and adopted outside the confines of proposing institutions. In the present study, we aimed to create a new, simple and useful classification system for colorectal liver metastases analogous to the Tumor Node Metastasis (TNM) classification system of International Union Against Cancer (UICC).

MATERIALS AND METHODS

Patients

Group 1: Between January 1, 1981 and March 31, 1997, 83 consecutive patients underwent partial hepatectomy for colorectal liver metastases at the First Department of Surgery (presently the Department of Surgical Oncology), Tokyo University Hospital. During the post-operative period, two patients died in the hospital. One died of secondary aspiration pneumonia and the other died of severe intra-abdominal sepsis (mortality rate: 3.1%). The remaining 81 patients were followed up either until death or their last outpatient visit up to December 31, 2002. The follow-up period ranged from 4 to 197 mo with a median of 53.0 mo. The demographic characteristics and tumor-

related features, which were statistically analyzed later, are summarized in Table 1.

Group 2: Between January 1, 1989 and December 31, 2003, 95 consecutive patients underwent partial hepatectomy for colorectal liver metastases at the Second Department of Surgery, Teikyo University Hospital. During the post-operative period, three patients died in the hospital. Two died of hepatic failure due to massive hepatectomy and the other died of severe intra-abdominal sepsis (mortality rate: 3.2%). The remaining 92 patients were followed up either until death or their last outpatient visit up to December 31, 2004. The follow-up period ranged from 4 to 110 mo with a median of 39.0 mo.

Every hepatectomy was considered curative since surgeons were confident of the complete macroscopic resection of hepatic tumors at the time of surgery. Hepatectomy was performed even in the presence of extrahepatic metastases if surgeons were reasonably assured of the complete macroscopic resection of extrahepatic metastases as well. After discharge from the hospital, the patients were closely monitored either at the outpatient clinic or at the affiliated institutions. Measurement of serum carcinoembryonic antigen (CEA) levels and ultrasonography were performed during post-operative visits at least once every two months in an effort to detect early recurrence. In addition, computed tomography was performed approximately twice a year. Almost all cases of cancer recurrence were diagnosed by these investigative tests. If the diagnosis was unclear, angiography and/or needle biopsy was performed, under ultrasonic guidance in an effort to confirm or rule out recurrence of the disease.

Prognostic factors

Eleven factors that were expected to influence the long-term prognosis were evaluated for statistical significance. These factors could only be determined preoperatively or during surgery (therapeutic factors were excluded and not considered). Factors that were considered included gender, age at hepatectomy (< 60 or ≥ 60 years), chronology of synchronous or metachronous hepatic metastases, and post-operative disease-free interval ≤ 1 year, the number of solitary or multiple metastatic nodule hepatic metastases, the maximum diameter of hepatic metastases (≤ 5 cm), unilobar or bilobar hepatic involvement, resectable extrahepatic metastasis (pulmonary metastases, localized peritoneal metastases, or hepatoduodenal lymph node metastases, each was completely resected), serum CEA levels at hepatectomy (less or higher than 10 times the upper level of normal), serosal exposure to the primary colorectal tumor, and regional lymph node metastases of the primary colorectal tumor.

Statistical analysis

Survival rates after hepatectomy were calculated using data obtained from patients by the Kaplan-Meier method. Only deaths attributable to recurrent cancer were treated as deaths due to disease. Patients who died of secondary or other causes without recurrence were treated as censored.

Prognostic variables concerned with cancer-related survival rate were entered into multivariate analysis. The Cox stepwise analysis proportional hazard regression model was used to select independent and significant prognostic variables. Stepwise variable selection was performed at a value of $P < 0.20$ level of significance. $P < 0.05$ was considered statistically significant.

Table 1 Prognostic factors entered into multivariate analysis based on proposed clinical and histopathological features

Variable	Patients (*n*)
Gender (male/female)	61/20
Age at hepatectomy (< 60/≥ 60)	32/49
Chronology of hepatic metastasis (synchronous/metachronous)	41/40
Disease free interval after colectomy (≤ 1 yr/> 1 yr)	56/25
Extrahepatic distant metastases (no/yes)	72/9
CEA (≤ 10 times of normal value/> 10 times of normal value)	57/24
Primary lesion	
Depth of invasion (up to subserosa/more)	57/24
Lymphnodemetastasi (no/yes)	39/42
Hepatic metastasis	
Number (single/multiple)	45/36
Maximum diameter (≤ 5 cm/> 5 cm)	59/22
Lobe involved (unilobar/bilobar)	64/17

RESULTS

Group 1

Significant prognostic factors: The overall 1-, 2-, 3-, 4-, and 5-year cancer-related survival rates after surgical resection in the 81 patients were 89.6%, 67.7%, 56.0%, 54.2%, and 49.6%, respectively (Figure 1A). The results of the multivariate analysis of the variables expected to influence cancer-related survival rate after surgical resection are provided in Table 1. Only variables selected by the stepwise analysis at a value of $P < 0.20$ level of significance, using the Cox proportional hazard regression model, are also shown in Table 2. Multiple tumors, tumor over 5 cm in diameter, and resectable extrahepatic metastases were significant and independent variables influenced cancer-related survival rate ($P < 0.05$). On the other hand, serosal exposure and regional lymph node metastases of the primary colorectal tumor, and recurrent hepatic metastases within one year after resection of the primary colorectal cancer including synchronous hepatic metastases, were the factors selected by stepwise analysis as the possible indication of poor prognosis, but they were not statistically significant ($P > 0.05$ or $P < 0.20$).

Classification of patients and survival: In devising our classification system, we considered three variables selected by the multivariate analysis, including the number and size of hepatic metastases (H-factor), and the presence of extrahepatic metastases (M-factor), which were resected completely. Results were shown as follows: H1: single metastasis with diameter ≤ 5 cm; H2: single metastasis with diameter > 5 cm or multiple metastases with diameter ≤ 5 cm; H3: multiple metastases with diameter > 5 cm; M0: extrahepatic metastasis (-); M1: extrahepatic metastasis (+, resectable). Staging system (A): stage I: H1 and M0; stage Ⅱ: H2 and M0; stage Ⅲ: H3 and M0; stage Ⅳ: H1-3 and M1; staging system (B): stage I: H1&M0; stage Ⅱ: H1 and M1 or H2 and M0; stage Ⅲ: H2 and M1 or H3 and M0; stage Ⅳ: H3 and M1

Table 2 Regression statistics for the stepwise cox proportional hazard model[1]

Variable	Parameter	*P*	Hazard Ratio (95% CI)
Diameter > 5 cm	1.35657	0.0013	3.883 (1.703-8.852)
Extrahep. Met (+).	1.19430	0.0133	3.301 (1.282-8.502)
Number ⩾ 2	0.85412	0.0265	2.349 (1.105-4.997)
D.F.interval < 1 yr	0.67838	0.1602	1.971 (0.765-5.080)
n (+) of primary	0.60034	0.1352	1.823 (0.829-4.007)
⩾ se (+) of primary	0.54877	0.1676	1.731 (0.794-3.774)

[1]Only those variables selected by the stepwise analysis, at the *P*-value of 0.20 level of significance, are shown. *n* = 81 (Tokyo Univ. 1981-1997). Age ⩾ 60, gender, synchronous, bilobar invasion, CEA; *P* > 0.2.

Staging system (A): The survival curves of patients in group 1 based on the staging system (A) are shown in Figure 1B. They were statistically significant ($P = 0.0057$). However, the survival curve of patients with stage Ⅳ cancer seemed to be better than that of those with stage Ⅲ cancer. The 5-year survival rates of patients with cancer in stage I ($n = 26$), stage Ⅱ ($n = 38$), stage Ⅲ ($n = 8$), and stage Ⅳ ($n = 9$) were 74.8%, 49.2%, 15.6%, and 25.0%, respectively. Their median survival time was 52, 18 and 19 mo, respectively, with that unsettled in stage I patients.

Staging system (B): The survival curves of patients in group 1 based on the staging system (B) are shown in Figure 1C, and the findings were statistically significant ($P = 0.0003$). The 5-year survival rates of patients with cancer in stage I ($n = 26$), stage Ⅱ ($n = 43$), and stage Ⅲ ($n = 12$) were 74.8%, 49.8%, and 9.5%, respectively. Their median survival time was 52, 52, and 18 mo, respectively. By coincidence, there were no patients with stage Ⅳ disease in this group.

Group 2

The overall 1-, 2-, 3-, 4-, and 5-year cancer-related survival rates after surgical resection in the 92 patients were 82.2%, 65.2%, 51.6%, 42.5%, and 40.0%, respectively (Figure 2A).

Staging system (A): The survival curves of 92 patients in group 2 based on the staging system (A) are shown in Figure 2B. They were statistically significant ($P < 0.0001$). However, the survival curve of patients with stage Ⅳ cancer seemed to be better than that of those with stage Ⅲ cancer. The 5-year survival rates of patients with cancer in stage I ($n = 24$), stage Ⅱ ($n = 44$), stage Ⅲ ($n = 8$), and stage Ⅳ ($n = 15$) were 75.0%, 44.5%, 0%, and 0%, respectively. Their median survival time was 46, 13.5, and 20 mo, respectively, with that unsettled in stage I patients.

Staging system (B): The survival curves of 92 patients in group 2 based on the staging system (B) are shown in Figure 2C, and findings were statistically significant ($P < 0.0001$). The 5-year survival rates of patients with cancer in stage I ($n = 24$), stage Ⅱ ($n = 48$), stage Ⅲ ($n = 16$), and stage Ⅳ ($n = 3$) were 75.0%, 43.0%, 0%, and 0%, respectively. Their median survival time was 41, 12, and 10 mo, respectively, with that unsettled in stage I patients.

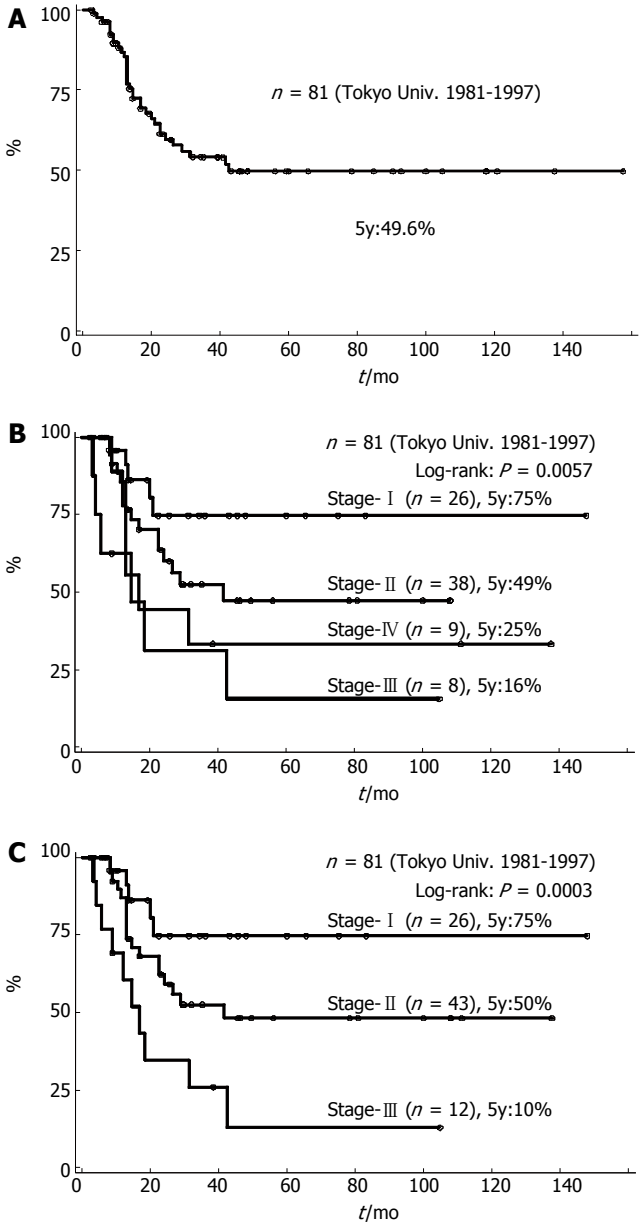

Figure 1 Kaplan-Meier cancer-related survival curve after hepatic resection for patients with colorectal liver metastases in group 1. **A**: Staging system (A) (log-rank test, $P = 0.0057$); **B**: staging system (B); **C**: stage Ⅰ through sage Ⅲ (log-rank test, $P = 0.0003$).

DISCUSSION

At present, there is no universally accepted classification system for patients with colorectal liver metastasis. Our first step in this investigation was to determine the important clinical and pathological variables that significantly influence prognosis following surgical resection of colorectal liver metastases. Many studies have identified factors which are thought to represent important prognostic determinants, including age at hepatectomy[4,7], sex[23], stage of the primary tumor[2,4,5,7,8,15,20,22,23,28,29] (including regional lymph node metastases and depth of invasion), synchronous or metachronous hepatic metastases[4,7,10,12,14-16,31], the number[4,7,10,11,17,22-25,31] and size[6,7,12,13,15,17,23,26-28] of hepatic metastases, the distribution of hepatic metastases[4,10,20,30], serum carcinoembryonic antigen (CEA) levels[7,11,29,31], extrahepatic metastases[2,8-10,12,14,22,23,26], type of hepatectomy[15], surgical margins[4,9,11,13,15-17,23,24,26], and adjuvant chemotherapy[12,32,33]. Therapeutic factors, such

Table 3 Our proposal staging system for colorectal liver metastases

Staging system
H-factor;
H1: Solitary&Diameter ≤ 5 cm
H2: Solitary&Diameter > 5 cm/Multiple&Diameter ≤ 5 cm
H3: Multiple&Diameter > 5 cm
M-factor;
M0: Extrahepatic metastasis (-)
M1: Extrahepatic metastasis (+): resectable
Staging;
Ⅰ: H1&M0
Ⅱ: H2&M0/H1&M1
Ⅲ: H3&M0/H2&M1
Ⅳ: H3&M1
[IVb: any H & unresectable extraheptic metastasis (+)]

as type of hepatectomy, surgical margin, and adjuvant chemotherapy, were excluded in the present statistical analysis, because these therapeutic factors are usually not considered in the classification system. At last three independent and significant determinants, such as multiple tumors, tumor larger than 5 cm in diameter, and macroscopically resected extrahepatic metastasis, were selected in the present study for devising a staging system.

To make a staging system, we considered the number and size of hepatic metastases as hepatic factors (H-factors) while the resected extrahepatic metastases as metastatic factor (M-factor). At first we considered M-factor even if extrahepatic metastases were resected, as an advanced state of disease, e.g. stage Ⅳ in the staging system (A). However, this classification did not seem logical since the prognosis of stage Ⅳ cancer patients was better than that of stage Ⅲ cancer patients. We therefore considered M-factor as one of the risk factors similar to H-factor variable in devising the staging system (B). Following this method, the patients were clearly classified according to their disease stage in group 1 and group 2. This result was reasonable because the risk ratio of M-factor was similar to that of H-factor variables, such as the number and size of hepatic metastases (3.301 *vs* 3.883/2.349) in the present study. Therefore, we propose a new staging system as shown in Table 3. It has not been our policy to perform hepatectomy for patients with unresectable extrahepatic metastases, such as diffuse lung metastases, diffuse peritoneal dissemination, and extensive lymph node metastases. In the present study, we included only the patients with extrahepatic metastases, who underwent hepatectomy as well as complete macroscopic resection of extrahepatic metastases. Therefore, patients with unresectable extrahepatic metastases should be classified as more advanced stages.

In conclusion, we have identified three important and independent prognostic determinants, namely multiple tumors, tumor larger than 5 cm in diameter, and resectable extrahepatic metastasis, using multivariate analysis from a retrospective review of patients who underwent surgical resection for colorectal liver metastases. At last we propose a new and simple staging system with these three determinants to classify the patients according to the long-term outcome after hepatectomy. We hope that it can promote a prospective study on the efficacy of some other therapies such as adjuvant chemotherapy. Although the present study population sample is small, our staging system is simpler and more useful than any other previous classification systems. Further investigation utilizing a larger patient population is necessary.

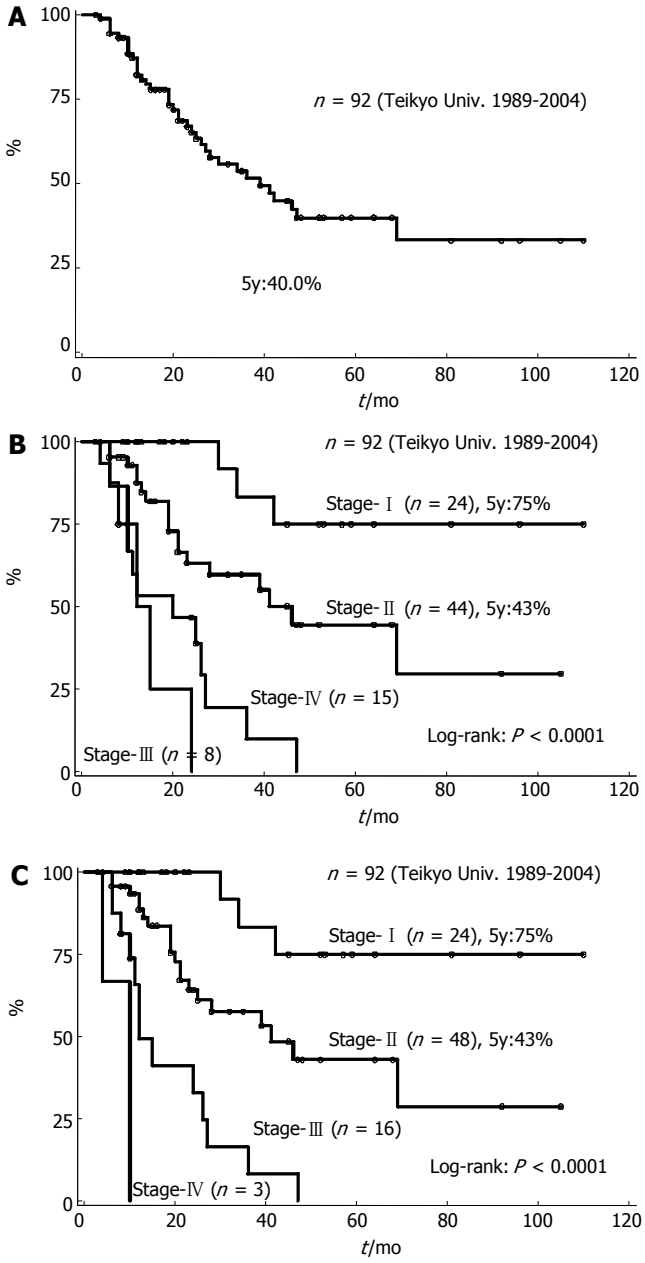

Figure 2 Kaplan-Meier cancer-related survival curve after hepatic resection for patients with colorectal liver metastases in group 2. **A**: Staging system (log-rank test, $P < 0.0001$); **B**: staging system; **C**: stage Ⅰ through stage Ⅲ (log-rank test, $P < 0.0001$).

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BASIC RESEARCH

Expression patterns and action analysis of genes associated with drug-induced liver diseases during rat liver regeneration

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Abstract

AIM: To study the action of the genes associated with drug-induced liver diseases at the gene transcriptional level during liver regeneration (LR) in rats.

METHODS: The genes associated with drug-induced liver diseases were obtained by collecting the data from databases and literature, and the gene expression changes in the regenerating liver were checked by the Rat Genome 230 2.0 array.

RESULTS: The initial and total expression numbers of genes occurring in phases of 0.5-4 h after partial hepatectomy (PH), 4-6 h after PH (G0/G1 transition), 6-66 h after PH (cell proliferation), 66-168 h after PH (cell differentiation and structure-function reconstruction) were 21, 3, 9, 2 and 21, 9, 19, 18, respectively. It is illustrated that the associated genes were mainly triggered at the initial stage of LR and worked at different phases. According to their expression similarity, these genes were classified into 5 types: only up-regulated (12 genes), predominantly up-regulated (4 genes), only down-regulated (11 genes), predominantly down-regulated (3 genes), and approximately up-/down-regulated (2 genes). The total times of their up- and down-expression were 130 and 79, respectively, demonstrating that expression of most of the genes was increased during LR, while a few decreased. The cell physiological and biochemical activities during LR were staggered according to the time relevance and were diverse and complicated in gene expression patterns.

CONCLUSION: Drug metabolic capacity in regenerating liver was enhanced. Thirty-two genes play important roles during liver regeneration in rats.

INTRODUCTION

The liver has a very strong capacity to regenerate[1]. Liver cells proliferate rapidly to compensate for lost liver tissues after liver injury or drug stimulus, which is called liver regeneration (LR)[2]. The LR process is usually categorized based on hepatic physiological activities divided into four stages: initiation phase [0.5-4 h after partial hepatectomy (PH)], transition from G0 to G1 (4-6 h after PH), cell proliferation (6-66 h after PH), cell differentiation and reorganization of the structure-function (66-168 h after PH)[3]. The process involves hepatic cell activation, dedifferentiation, proliferation and its regulation, redifferentiation, structure-functional reorganization[4].

Liver is a vital organ of drug metabolism[5]. Disorder of drug metabolism in liver could cause drug-induced liver diseases[6]. It is indicated that 182 genes are associated with drug-induced liver diseases. In addition, there are gene-gene, protein-protein, gene-regulator, and protein-regulator interactions. It is hardly possible to highlight the role of the genes in LR unless gene expression profiles is analyzed with high-throughput[7,8]. Therefore, we used the Rat Genome 230 2.0 array containing 84 genes associated with drug-induced liver diseases to detect gene expression changes after PH, finding that 32 of them were associated with LR, and analyzed these genes expression changes, patterns and actions during LR primarily.

MATERIALS AND METHODS

Regenerating liver preparation

Healthy SD rats weighing 200-250 g were obtained from the Animal Center of Henan Normal University. The rats were separated into groups at random and each group included 6 rats (male:female = 1:1). PH was performed according to Higgins and Anderson[9], the left and middle

lobes of the liver were removed. Rats were killed by cervical vertebra dislocation at 0.5, 1, 2, 4, 6, 8, 12, 16, 18, 24, 30, 36, 42, 48, 54, 60, 66, 72, 96, 120, 144 and 168 h after PH and the regenerating livers were observed at corresponding time points. The livers were rinsed three times in PBS at 4℃, then 100-200 mg liver from the middle parts of the right lobe, six samples of each group were collected, wrer mixed with 1-2 g (0.1-0.2 g × 6) total liver tissues, and stored at -80℃. The sham-operation (SO) groups were treated the same with the PH group except that the liver lobes were unremoved. The laws of animal protection of China were enforced strictly.

RNA isolation and purification

Total RNA was isolated from frozen livers according to the manual of the Trizol kit (Invittrogen)[10] and purified based on the RNeasy mini-kit (Qiagen)[11]. Total RNA samples exhibited a 2:1 ratio of 28S to 18S rRNA intensities by agarose electrophoresis (180V, 0.5h). Total RNA concentration and purity were estimated by optical density measurement at 260/280 nm[12].

cDNA, cRNA synthesis and purification

As a template, 1-8 μg total RNA was used for cDNA synthesis. cDNA and cRNA synthesis was proceeded by the established method of Affymetrix[13]. cRNA labeled with biotin was synthesized using 12 μL of the above synthesized cDNA as the template, and cDNA and cRNA were purified[13]. Concentration, purity and quality of cDNA and cRNA were measured by the same method mentioned above[12].

cRNA fragmentation and microarray detection

Fifteen μL (1 μg/μL) cRNA incubated with 5 × fragmentation buffer at 94℃ for 35 min was digested into 35-200 bp fragments. The hybridization buffer was added to the prehybridized Rat Genome 230 2.0 microarray produced by Affymetrix, then hybridization was carried out for 16 h at 45℃ on a rotary mixer at 60 rpm. The microarray was washed and stained by GeneChip fluidics station 450 (Affymetrix Inc., USA). The chips were scanned by GeneChip Scan 3000 (Affymetrix Inc., USA), and the signal values of gene expression were observed[14].

Microarray data analysis

The normalized signal values, signal detections (P, A, M) and experiment/control (Ri) were obtained by quantifying and normalizing the signal values using GCOS1.2[14].

Normalization of microarray data

To minimize error in the microarray analysis, each analysis was performed three times by Rat Genome 230 2.0 microarray. Results with a total ratio was maximal (R^m) and when the average of three housekeeping genes (β-actin, hexokinase and glyceraldehyde-3-phosphate dehydrogenase) approached 1.0 (R^h), it was taken as a reference. The modified data were generated by applying a correction factor (R^m/R^h) multiplying the ratio of every gene in R^h at each time point. To remove spurious gene expression changes resulting from errors in the microarray analysis, the gene expression profiles at 0-4 h, 6-12 h and 12-24 h after PH were reorganized by NAP software (normalization analysis program) according to the cell cycle progression of the regenerating hepatocytes. Data statistics and cluster analysis were done using GeneMath, GeneSpring, Microsoft Excel software[14-16].

Identification of genes associated with liver regeneration

The nomenclature of a liver disease (e.g. drug-induced liver diseases) was adopted from the GENEONTOLOGY database (www.geneontology.org), and input into the databases at NCBI (www. ncbi.nlm.nih.gov) and RGD (rgd. mcw.edu) to identify the rat, mouse and human genes associated with the above liver diseases. Then the genes associated with the drug-induced liver diseases were collated. The results of this analysis were codified, and compared with the results obtained for human and mouse searches in order to identify the difference of human and mouse genes from rats. In comparison to these genes with the analysis output of the Rat Genome 230 2.0 array, the genes, showing a greater than twofold change in expression level as meaningful expression changes[17] , were referred to as rat homologous genes or rat specific genes associated with drug-induced liver diseases. Genes, which displayed reproducible results with three independent analyses with the chip and showed a greater than twofold change in expression level in at least one time point during liver regeneration with significant difference ($0.01 \leqslant P < 0.05$) or extremely significant difference ($P \leqslant 0.01$) between PH and SO, were referred to as associated with liver regeneration.

RESULTS

Expression changes of genes associated with drug-induced liver diseases during LR

According to the data from databases at NCBI, GENEMAP, KEGG and BIOCARTA, 182 genes were associated with drug-induced liver diseases. Among them, 84 genes were contained in the Rat Genome 230 2.0 array. Thirty-two of them revealed meaningful changes in expression at least at one time point after PH. There was significant difference or extremely significant difference in expression between PH and SO, and reproducible results were obtained with three analyses with Rat Genome 230 2.0 array, suggesting that the genes were associated with LR (Table 1). The analysis indicated that 12 genes were up-, 11 genes down-, and 9 were up/down-regulated during liver regeneration. Total expression times of up- and down-expressed genes were 130 and 79, respectively (Figure 1A). At the initial stage of liver regeneration (0.5-4 h after PH), 13 genes displayed up-, 7 genes down-, 1 gene up/down-regulation; at the transition phase from G_0 and G_1 (4-6 h after PH), 10 genes revealed up-, 2 genes down-regulation; at cell proliferation phase (6-66 h after PH), 12 genes showed up-, 8 genes down-, 5 genes up/down-regulation; and at cell differentiation and structure-function reorganization phase (66-168 h after PH), 9 genes displayed up-, 10 genes down-, 5 genes up/down-regulation (Figure 1B).

Table 1 Expression abundance of 32 genes associated with drug-induced liver diseases during liver regeneration

Gene	Recovery time (h) after partial hepatectomy (PH)																						
Abbr.	0	0.5	1	2	4	6	8	12	16	18	24	30	36	42	48	54	60	66	72	96	120	144	168
1 Cholestasis																							
Ace	1	0.80	**0.48**	1.29	0.88	1.39	0.82	1.14	1.16	1.00	0.88	0.93	1.18	1.03	1.18	1.03	1.39	0.56	0.71	0.78	0.75	0.56	**0.47**
Akt1	1	0.81	0.76	0.98	0.86	1.01	0.87	1.11	1.12	0.62	0.71	*3.91*	0.65	*2.60*	0.53	0.57	0.68	1.10	0.97	1.17	1.02	0.94	1.01
Apoe	1	0.93	1.24	0.87	0.93	1.07	0.95	0.76	**0.15**	0.93	1.08	**0.13**	0.93	**0.17**	1.00	1.00	0.93	1.07	1.20	**0.33**	1.41	1.23	1.07
Bdnf	1	0.97	**0.46**	1.98	1.43	1.04	1.04	*2.00*	*2.08*	0.63	0.58	*2.12*	0.52	1.43	**0.45**	1.25	*2.50*	1.06	1.32	*2.56*	**0.39**	0.52	**0.50**
Cyp2d6	1	0.76	**0.33**	1.62	1.00	1.15	1.35	1.62	1.14	1.15	1.24	0.60	0.57	0.74	0.81	0.81	0.66	1.73	0.65	1.07	0.66	**0.44**	**0.50**
Ephx1	1	1.52	1.53	1.32	0.93	1.00	0.63	**0.41**	0.86	0.76	0.62	0.97	*2.46*	1.12	*2.14*	1.74	*2.00*	*2.82*	*2.77*	0.54	1.74	1.62	1.62
Il6	1	1.87	1.08	*3.25*	*3.25*	*3.03*	*3.81*	1.32	0.99	*3.25*	1.87	0.91	**0.50**	1.28	*2.83*	1.87	*2.30*	0.87	**0.28**	*6.06*	0.81	1.52	0.93
Mmp9	1	*2.05*	*2.16*	*2.10*	*2.07*	1.37	*2.81*	*2.75*	*2.13*	1.12	1.58	1.27	1.83	*5.89*	1.00	1.20	1.15	1.16	1.21	*9.54*	*2.22*	**0.48**	*2.03*
Slc22a1	1	0.62	0.88	0.76	0.66	0.66	0.59	0.71	0.86	0.81	0.82	1.20	0.71	1.47	**0.41**	0.71	0.62	1.31	1.05	1.23	0.81	0.76	0.71
Slc22a2	1	1.23	1.01	**0.50**	0.93	0.87	**0.39**	**0.38**	**0.33**	**0.38**	**0.33**	**0.08**	0.87	0.69	1.07	1.07	1.62	**0.18**	0.98	**0.38**	0.66	1.15	1.32
2 Fatty liver																							
Esr1	1	1.07	0.62	*3.48*	*4.92*	*4.92*	1.17	1.41	1.31	*2.14*	1.08	1.29	1.23	1.37	1.74	2.64	4.29	1.51	1.48	1.23	*4.59*	*6.06*	*2.30*
Fabp1	1	0.87	1.16	0.81	0.81	1.00	0.77	0.66	1.40	0.87	0.94	1.29	0.87	1.12	0.87	0.93	0.93	1.07	1.29	**0.50**	1.23	1.00	0.93
Hiat1	1	0.93	1.43	1.07	1.07	1.32	1.90	1.15	1.14	1.15	1.33	**0.10**	1.00	**0.14**	1.41	1.07	0.93	1.41	1.20	**0.15**	1.32	1.32	1.32
Hsd11b1	1	*4.92*	*6.13*	*2.64*	*4.92*	*2.83*	*3.81*	1.07	0.99	*2.14*	*3.50*	1.04	0.87	0.97	*2.83*	1.87	*2.46*	0.93	*2.77*	0.93	*3.73*	*5.28*	*4.92*
Il5	1	1.15	0.67	1.00	0.76	0.76	1.26	1.52	0.86	*2.83*	1.52	0.85	1.32	1.28	*3.03*	*3.25*	*3.48*	1.51	1.70	1.07	0.93	1.32	1.32
Mthfr	1	**0.47**	**0.44**	1.87	*3.25*	*2.64*	*3.19*	*3.73*	1.50	**0.50**	**0.38**	1.48	0.66	1.69	0.66	0.71	0.87	**0.46**	**0.40**	*2.00*	0.57	0.66	**0.50**
Tnf	1	0.87	0.62	1.23	1.41	1.15	1.17	0.93	0.93	0.71	0.66	0.79	1.41	0.85	1.62	1.07	1.15	0.93	0.52	1.23	*3.25*	0.81	0.71
Trp63	1	*3.00*	*2.44*	*2.40*	1.06	0.97	1.70	*2.03*	1.58	1.81	1.35	0.84	1.19	1.33	1.55	2.12	1.62	1.23	1.12	1.11	0.74	1.19	0.90
Ugt1a1	1	0.93	1.43	1.28	1.23	1.15	0.99	0.71	*2.06*	1.04	0.88	1.36	1.04	1.25	0.95	0.95	1.08	1.51	1.34	0.69	1.42	1.12	1.08
3 Liver ischemic injury																							
Ces2	1	0.81	1.08	1.32	1.32	1.23	1.44	1.00	*2.81*	1.23	1.42	0.85	1.62	*2.23*	1.74	1.62	1.52	*3.24*	*2.77*	1.23	1.87	1.41	1.52
Ces3	1	1.74	*3.29*	*2.46*	*3.03*	*3.03*	*2.88*	1.32	0.70	0.87	1.00	0.64	**0.44**	0.60	**0.27**	**0.44**	0.57	**0.33**	1.48	0.71	1.23	1.62	1.62
Creb1	1	0.93	1.01	0.93	**0.50**	1.00	0.55	0.66	1.06	1.23	0.94	0.91	0.87	0.91	0.87	1.41	1.15	1.14	0.85	0.93	1.23	1.07	1.00
Fos	1	*28.43*	*16.11*	*8.54*	*3.07*	*4.82*	*2.07*	*3.72*	*3.50*	*2.83*	*2.63*	*3.21*	*1.77*	*2.03*	*2.76*	1.65	1.69	1.90	1.71	1.12	*2.44*	0.88	1.00
Per1	1	*3.03*	*3.49*	*4.29*	*3.91*	*3.73*	*3.09*	0.93	1.61	**0.38**	0.71	1.38	**0.44**	1.81	**0.44**	**0.33**	**0.47**	0.75	0.79	1.15	*3.48*	*4.00*	*3.48*
4 Drug hepatitis																							
Bcl2	1	0.82	0.64	1.12	1.00	1.19	1.17	1.15	0.85	**0.33**	**0.37**	1.80	**0.32**	0.79	**0.44**	**0.39**	**0.46**	0.81	**0.38**	0.84	0.50	0.68	0.64
Cyp2d6	1	0.76	**0.33**	1.62	1.00	1.15	1.35	1.62	1.14	1.15	1.24	0.60	0.57	0.74	0.81	0.81	0.66	1.73	0.65	1.07	0.66	**0.44**	**0.50**
Egr1	1	*17.15*	*18.59*	*13.93*	*2.30*	*2.00*	*2.68*	*3.48*	0.86	1.62	*2.84*	0.64	*3.03*	0.56	*2.83*	*3.25*	*3.73*	*4.58*	*2.25*	0.66	*3.03*	1.00	0.93
Esr1	1	1.07	0.62	*3.48*	*4.92*	*4.92*	1.17	1.41	1.31	*2.14*	1.08	1.29	1.23	1.37	1.74	*2.64*	*4.29*	1.51	1.48	1.23	*4.59*	*6.06*	*2.30*
Gstm1	1	1.32	*2.17*	1.32	1.52	1.52	1.26	0.66	1.61	1.23	1.42	1.12	1.23	1.69	1.41	1.52	1.52	1.73	*2.10*	1.07	1.62	1.52	1.32
Il5	1	1.15	0.67	1.00	0.76	0.76	1.26	1.52	0.86	*2.83*	1.52	0.85	1.32	1.28	*3.03*	*3.25*	*3.48*	1.51	1.70	1.07	0.93	1.32	1.32
Mthfr	1	**0.47**	**0.44**	1.87	*3.25*	*2.64*	*3.19*	*3.73*	1.50	**0.50**	**0.38**	1.48	0.66	1.69	0.66	0.71	0.87	**0.46**	**0.40**	*2.00*	0.57	0.66	**0.50**
Nat2	1	1.00	1.89	1.07	*2.30*	1.32	1.09	1.41	0.99	*3.25*	*4.02*	1.29	1.07	1.04	*3.48*	*2.83*	*3.25*	*2.63*	1.70	1.41	*3.48*	1.74	*2.46*
Pten	1	0.66	1.08	0.93	**0.50**	**0.47**	0.72	0.76	0.86	1.23	1.33	0.79	1.41	1.04	1.74	1.32	1.23	1.62	1.12	1.41	1.52	1.32	1.23
Ptgs2	1	0.57	0.90	*2.14*	1.23	1.41	1.17	1.41	1.31	0.62	0.66	0.85	**0.12**	1.12	**0.47**	0.71	1.15	**0.31**	0.69	1.00	**0.44**	**0.33**	**0.47**
Trp63	1	*3.00*	*2.44*	*2.40*	1.06	0.97	1.70	2.03	1.58	1.81	1.35	0.84	1.19	1.33	1.55	*2.12*	1.62	1.23	1.12	1.11	0.74	1.19	0.90
5 Granulomatous disease of the liver																							
Cyp2d6	1	0.76	**0.33**	1.62	1.00	1.15	1.35	1.62	1.14	1.15	1.24	0.60	0.57	0.74	0.81	0.81	0.66	1.73	0.65	1.07	0.66	**0.44**	**0.50**
6 Peliosis hepatis																							
Erbb2	1	**0.50**	**0.10**	1.74	0.66	0.62	**0.34**	0.62	1.40	**0.29**	**0.17**	1.12	**0.41**	1.47	**0.50**	**0.41**	**0.47**	**0.15**	**0.15**	1.15	**0.25**	**0.23**	**0.31**
Sult1a1	1	1.62	*2.17*	1.41	1.52	1.41	1.26	0.76	**0.22**	1.00	1.24	1.12	1.15	**0.49**	1.87	1.32	1.41	1.62	1.70	0.71	1.74	1.62	1.62

Italic numbers: Genes are up-regulated more than twofold; Bold numbers: Genes are down-regulated more than twofold.

Initial expression time of genes associated with drug-induced liver diseases during LR

At each time point of LR, the numbers of initial up-, down-regulation and total up-, down-regulation genes were in the sequence: both 6 and 2 at 0.5 h; 3, 3 and 9, 5 at 1 h; 3, 1 and 10, 1 at 2 h; 1, 2 and 10, 2 at 4 h; 0, 0 and 8, 1 at 6 h; 0, 0 and 8, 2 at 8 h; 0, 1 and 6, 2 at 12 h; 2, 1 and 5, 3 at 16 h; 1, 1 and 6, 5 at 18 h; 0, 0 and 4, 4 at 24 h; 1, 1 and 3, 3 at 30 h; 0, 0 and 2, 6 at 36 h; 0, 0 and 4, 3 at 42 h; 0, 1 and 7, 7 at 48 h; 0, 0 and 5, 4 at 54 h; 0, 0 and 8, 3 at 60 h; 0, 0 and 4, 5 at 66 h; 0, 0 and 5, 4 at 72 h; 0, 1 and 4, 4 at 96 h; 1, 0 and 8, 3 at 120 h; 0, 0 and 3, 4 at 144 h , 0, 0 and 5, 6 at 168 h (Figure 2).

Expression similarity and time relevance of genes associated with drug-induced liver diseases during LR

Thirty-two genes mentioned above during LR could be characterized based on their similarity in expression as follows: only up-, predominantly up-, only down-, predominantly down-, and up-/down-regulated, involving 13, 4, 11, 3 and 2 genes, respectively (Figure 3). They could also be classified according to the time relevance into 15 groups, including 0.5 and 1 h, 2 h, 4 and 6 h, 8 h, 12 h, 16 h, 18 and 24 h, 30 h, 36 h, 42 and 96 h, 48 h, 54 h and 60 h, 66 and 72 h, 120 h, 144 and 168 h. Their times of up- and down-regulation genes were respectively 15 and 7, 10 and 1, 18 and 3，8 and 2, 6 and 2，6 and 3, 10 and 9, 3 and 3, 6 and 2, 8 and 7, 7 and 7, 13 and 7, 9 and 9, 8 and 3, 8 and 10 (Figure 3).

Expression patterns of genes associated with drug-induced liver diseases during LR

Thirty-two genes mentioned above during LR might be

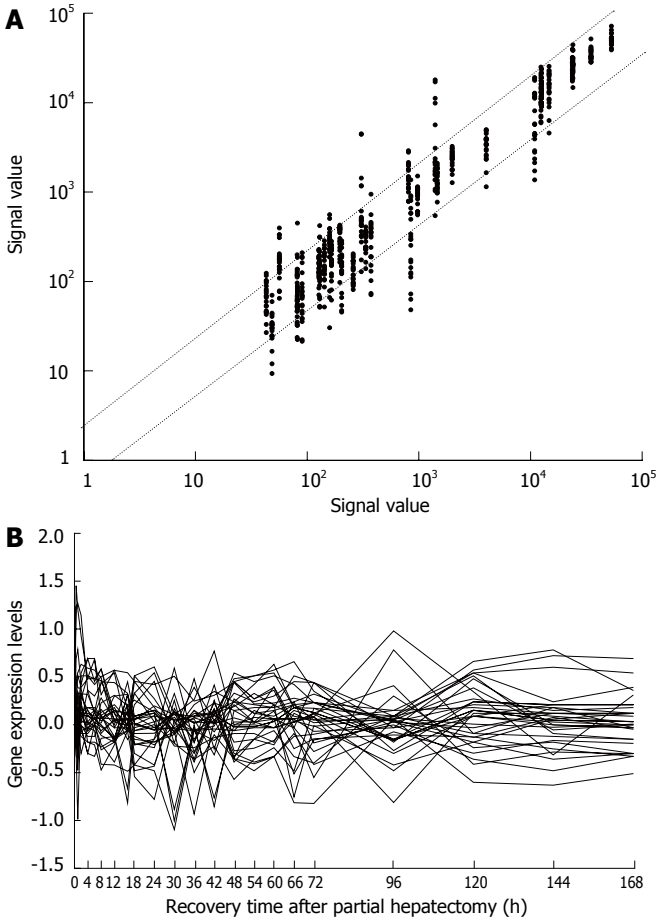

Figure 1 Expression profiles of 32 genes associated with drug-induced liver diseases during liver regeneration. **A**: The abundance and frequency of gene expression, each point represents the signal value of one gene at corresponding time point. The dots above bias represent the genes up-regulated by more than twofold, those under bias represent the genes down-regulated by more than twofold, and the ones between biases represent the genes meaningless alteration in expression. The farer the genes from the bias, the greater the folds of gene change; **B**: The expression changes of genes associated with LR.

categorized according to the changes in expression into 20 types of patterns: (1) up-regulation at one time point, i.e. at 16, 120 h after PH, (Figure 4A), 2 genes; (2) up- at two time point, i.e. at 1 and 72 h, 30 and 42 h, (Figure 4B), 2 genes; (3) up- at one time point/phase, i.e. at 18 and 48-72 h (Figure 4C), 1 gene; (4) up- at one time point/two phases (Figure 4C), 1 gene; (5) up- at one time point/three phases (Figure 4C), 1 gene; (6) up- at two time points/one phase (Figure 4D), 2 genes; (7) up- at three time points/two phases (Figure 4E), 2 genes; (8) up- at three time points/phases (Figure 4D), 1 gene; (9) down- at one time point, at 0.5, 48 or 96 h (Figure 4F), 3 genes; (10) down- at two time points, i.e. at 1 and 168 h (Figure 4G), 1 gene; (11) down- at three time points(Figure 4G), 1 gene; (12) down- at more time points (Figure 4G), 1 gene; (13) down- at one phase, i.e. at 1-6 h (Figure 4H), 1 gene; (14) down- at one time point/phase, i.e. at 1 and 144-168 h (Figure 4H), 1 gene; (15) down- at two time points/phases (Figure 4H), 1 gene; (16) down- at two time points/four phases (Figure 4H), 1 gene; (17) down- at three time points/one phase (Figure 4H), 1 gene; (18) predominantly up- (Figure 4I), 4 genes; (19) predominantly down- (Figure 4J), 3 genes; (20) up/down- approximately (Figure 4K), 2 genes.

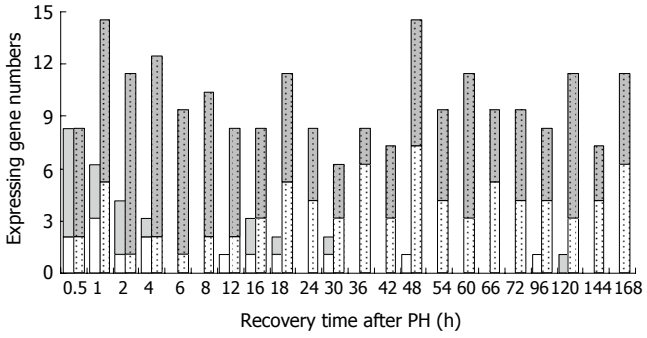

Figure 2 The initial and total expression profiles of 32 genes associated with drug-induced liver diseases at each time point of liver regeneration. Blank bars: Initial expressing gene number; Dotted bars: Total expressing; Grey bars: Up-regulated; White bars: Down-regulated.

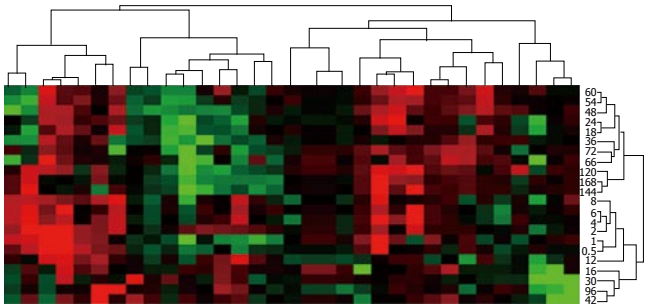

Figure 3 Expression similarity and time relevance clusters of 32 genes associated with drug-induced liver diseases during liver regeneration. Detection data of Rat Genome 230 2.0 array were analyzed by H-clustering. Red: Up-regulation genes; Green: Down-regulation; Black: No-sense in expression change; The upper and right trees respectively show expression cluster and time series cluster.

DISCUSSION

In this paper, the roles of 84 genes associated with drug-induced liver diseases during liver regeneration were analyzed. Of the 36 genes associated with drug-induced abnormality of cell proliferation and apoptosis, cocaine addiction-associated cAMP responsive element binding protein 1 (CREB1)[18] and estradiol-induced interleukin 6 (IL6)[19] were related to liver regeneration initiation[20]. Cocaine-induced V-fos FBJ murine osteosarcoma viral oncogene homolog (FOS)[21], troglitazone-induced early growth response 1 (EGR-1)[22], prostaglandin-endoperoxide synthase 2 (PTGS2) repressed by cyclophosphamide[23], estradiol-activated Akt (v–akt) murine thymoma viral oncogene homolog 1 (AKT1)[24] and estradiol-induced brain derived neurotrophic factor (BDNF)[25] all promote cell growth or cell division[26,27]. Valproic acid-restrained estrogen receptor 1 (ESR1)[28] inhibits cell division[29]. Period homolog 1 (Drosophila) (PER1) promotes apoptosis[30]. Cyclophosphamide-induced B-cell leukemia/lymphoma 2 (BCL2) restrains apoptosis[31]. Diethylstilbestrol-restrained transformation related protein 63 (TRP63)[32] is associated with differentiation[33]. Estradiol-induced matrix metallopeptidase 9 (MMP9)[34] is involved in the breakdown of extracellular matrix. Indomethacin-induced phosphatase and tensin homolog (PTEN)[35] blocks tumor cell proliferation and migration[36].

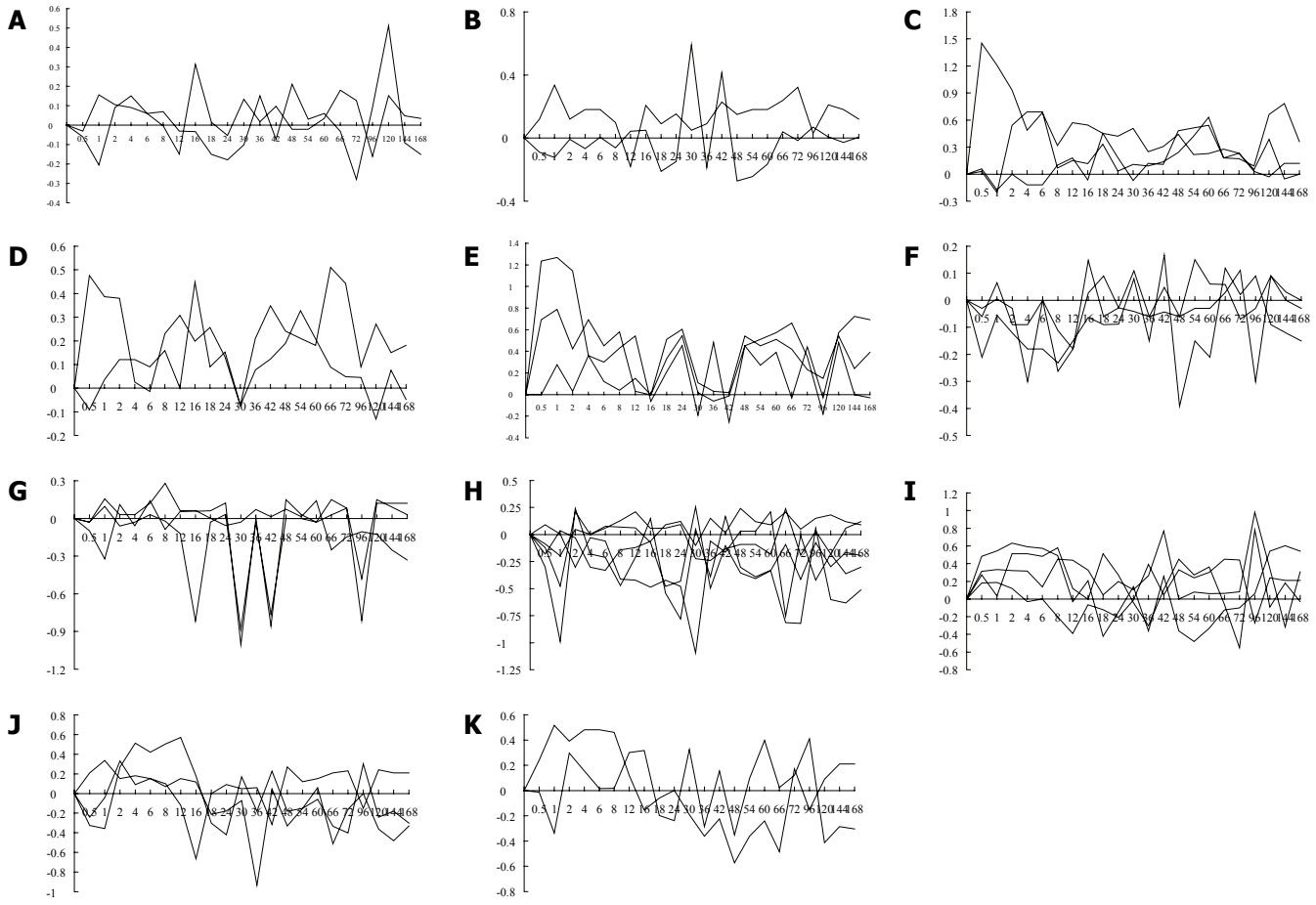

Figure 4 Expression patterns of 32 genes associated with drug-induced liver diseases during liver regeneration. These genes exhibit 20 types of expression patterns. **A-E**: Up-regulation in expression; **F-H**: Down-regulation; **I-J**: Up/down-regulation mixed. X-axis represents recovery time after PH (h), Y-axis shows logarithm ratio of the signal values of genes at each time point to the control.

Angiotensin I converting enzyme (ACE), whose activity is inhibited by captopril[57], participates in the control of blood pressure. The sameness or similarity in some time points, and the difference in other points of meaningful expression changes of these genes during LR may indicate that they regulate the mass of regenerating liver together.

Of the 21 genes associated with drug-induced disorder of lipid metabolism or amino-acid metabolism, estradiol-induced apolipoprotein E (APOE)[38] and fatty acid binding protein 1, liver (FABP1) play a part in the metabolism and transport of lipid[39,40]. One of the tamoxifens' target proteins: epoxide hydrolase 1, microsomal (EPHX1)[41], and tetracyclin-induced tumor necrosis factor (TNF)[42] participate in lipid metabolism, and 5, 10-methylenetetrahydrofolate reductase (MTHFR)[43] plays a role in methionine biosynthesis. That meaningful expression changes of these genes are the same or similar in some time points, then different in other points during LR perhaps regulate the metabolism of lipid and/or amino-acid together.

Of the 27 genes associated with drug metabolism disorder, six genes including solute carrier family 22 member 1, 2, (SLC22A1, SLC22A2), UDP glucuronosyltransferase 1 family A1 (UGT1A1), glutathione S-transferase M1 (GSTM1), amitriptyline-restrained cytochrome P450 family 2 subfamily D 6 (CYP2D6)[44] and sulfotransferase family cytosolic 1A phenol-preferring member 1 (SULT1A1) are involved in drug metabolism[45-47]. Hippocampus abundant gene transcript 1 (HIAT1) is responsible for transmembrane of tetracyclin[48]. N-acetyltransferase 2 (NAT2) catalyzes decomposition of isoniazid. Hydroxysteroid (11-β) dehydrogenase 1 (HSD11B1) can inactivate cortisol[49]. Carboxylesterase 2, 3 (CES2, CES3) catalyze the hydrolysis of fatty acids and cocaine[50]. Interleukin 5 (IL5) is associated with corticosteroid resistance[51] and inflammation[52]. V-erb-b2 erythroblastic leukemia viral oncogene homolog 2 (ERBB2) can impede the function of tamoxifen[53]. The expression changes of the genes mentioned above were the same or similar at some time points and different at other time points during LR, speculating that they promote drug metabolism together.

In conclusion, some genes associated with drug-induced liver diseases are up-regulated and the others are down-regulated during liver regeneration. In liver regeneration, some drug-induced liver diseases-related genes regulate the liver cell number by adjusting cell proliferation and apoptosis, some control lipid metabolism or amino acid metabolism, and others participate and modulate drug metabolism, demonstrating that they are closely in line with liver regeneration. We will use northern blotting, protein array, RNA interference etc. to further confirm the above results at the cell level in the future.

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CLINICAL RESEARCH

Meta-analysis on inoperable pancreatic cancer: A comparison between gemcitabine-based combination therapy and gemcitabine alone

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Abstract

AIM: To compare gemcitabine-based combination therapy and gemcitabine (GEM) alone in patients with advanced pancreatic cancer (APCa) through meta-analysis.

METHODS: MEDLINE and EMBASE searches were supplemented by information from trial registers of randomized controlled trials (RCTs) for GEM-based combination therapy and GEM alone for APCa. A quantitative meta-analysis was carried out by two reviewers based on the inclusion criteria from all available RCTs. The meta-analysis involved overall survival (OS), objective remission rate (ORR), clinical benefit rate (CBR), time to progress/progress free survival (TTP/PFS) and toxicity.

RESULTS: The meta-analysis included 22 RCTs. There was significant improvement in the GEM combination group with regard to the 6-mo survival rate (RD = 0.04, 95% CI 0.01-0.06, P = 0.008), 1-year survival rate (RD = 0.03, 95% CI 0.01-0.05, P = 0.01), ORR (RD = 0.04, 95% CI 0.01-0.07, P = 0.02), CBR (RD = 0.10, 95% CI 0.02-0.17, P = 0.01) and 6-mo TTP/PFS (RD = 0.07, 95% CI 0.04-0.10, P < 0.00001). However, the Grade 3-4 toxicity set by WHO was higher for the GEM combination group for neutropenia (RD = 0.05, 95% CI 0.01-0.10, P = 0.02), thrombocytopenia (RD = 0.05, 95% CI 0.02-0.08, P = 0.002) and vomiting/nausea (RD = 0.03, 95% CI 0.00-0.05, P = 0.02).

CONCLUSION: GEM-based combination therapy may improve the overall survival and palliation in optimal patients with APCa as compared with GEM alone.

Key words: Chemotherapy; Pancreatic cancer; Meta-analysis; Gemcitabine

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INTRODUCTION

Gemcitabine (GEM) monotherapy currently is considered as a standard treatment for patients with advanced pancreatic cancer (APCa). However, patients treated with GEM alone have poor prognoses, and their overall median survival (OS) was only 5.65 mo[1]. Attempts have been made to increase the objective remission rate (ORR) and survival of APCa patients, in particular, by exploring the effects of the combined GEM with other drugs. In many phase Ⅱ studies, GEM combinations have improved ORR and OS. Based on these results, many prospective, randomized phase Ⅲ trials comparing GEM used in combination and alone have been carried out. But these trials had different results and the population enrolled is small. Therefore, the NCCN guidelines (National Comprehensive Cancer Network, v.2.2006) indicate that GEM-based combination therapy may be an optimal treatment for APCa patients with a good performance status, including GEM + cisplatin (DDP), GEM + oxaliplatin, GEM + capecitabine, GEM + erlotinib and so on. But these guidelines were based on low level evidence including clinical experience (category 2A). The role of GEM-based combination therapy for the treatment of APCa is still unclear. We therefore, conducted a systematic review and quantitative meta-analysis to evaluate the available evidence from the relevant randomized trials.

MATERIALS AND METHODS

Literature search

We carried out a comprehensive search of literature

with MEDLINE (1966-2006), EMBASE (1966-2006), CBMdisc (1981-2006), ASCO Abstracts (1995-2005) and EBM Reviews (Cochrane Database of Systematic Reviews 1st Quarter, 2006) ACP Journal Club (1991-2006), (Database of Abstracts of Reviews of Effects 1st Quarter 2006), Cochrane Central Register of Controll ed Trials (1st Quarter, 2006), using the terms: 'pancreas', 'pancreatic cancer', 'pancreatic carcinoma, 'pancreatic adenocarcinoma', 'pancreatic neoplasms', 'gemzar', ' gemcitabine' (no limit to language). Date of last search: April 26, 2006.

Selection criteria

Study design: Trials should be prospective, properly randomized and well designed, which were matched for age, stage, performance status, *etc*.

Study population: Patients with APCa, as well as those with locally advanced, or metastatic disease, were included in the study. Patients eligible for the study were required to have histologically or cytologically proved pancreatic cancer. Furthermore, they should have a baseline Karnofsky performance status of $\geqslant$ 50% (or Eastern Cooperative Oncology Group performance status < 2) and adequate hematological, renal, cardiac and hepatic function. Patients with estimated life expectancy of at least 12 wk, should have received no chemotherapy, radiotherapy and other antitumor therapy in the 6 mo prior to the study entry.

Intervention: The treatment group received GEM-based combination therapy, and the control group received GEM alone.

Outcome: The primary outcome measurement was OS, followed by ORR and toxicity. The follow-up rate should be above 95%.

Data collection and analysis: Two reviewers assessed the abstracts identified from the defined sources. Both reviewers independently selected trials for inclusion according to prior agreement regarding the study population and the intervention. If one of the reviewers concluded an abstract to be eligible, the full text of article was retrieved and reviewed in detail by both reviewers. Missing data from the primary study reports was requested from the investigators. If the same trial appeared on different publications, the final data of the trial was chosen. Methodologic quality of the trials was assessed using a validated scale (range, 0 to 5) applied to items that influence the intervention efficacy. It was reported by Jadad *et al*[2] that the scale consisted of items pertinent to randomization, masking, dropouts, and withdrawals. The following information was obtained from each trial: year of publication, number of patients, performance status, chemotherapy regimen, objective response rate, overall survival, progress free survival, clinical benefit, toxicity, etc. For response assessment, we used trials that included patients with measurable or assessable diseases, and that were analyzed mainly with WHO's criteria. Toxicity profiles were reported according to the WHO's criteria.

Statistical analysis

The primary end point was a 6-mo survival rate after randomization. The other end points were 1-year survival rate, ORR, CBR, 6-mo TTP/PFS rate and adverse effects. All variables were defined as dichotomous data (e.g., 6-mo survival rate used variables as follows: the alive or dead at 6 mo after randomization). We standardized the therapeutic results by obtaining the risk difference (RD) between the GEM combination group and the GEM alone group. Publication bias was examined using a funnel plot[3]. We looked for heterogeneity among the trials based on Cochran's χ^2 test. All analyses were performed strictly with RevMan software (version 8.2, Cochrane). *P* value less than 0.05 was considered as significant in difference.

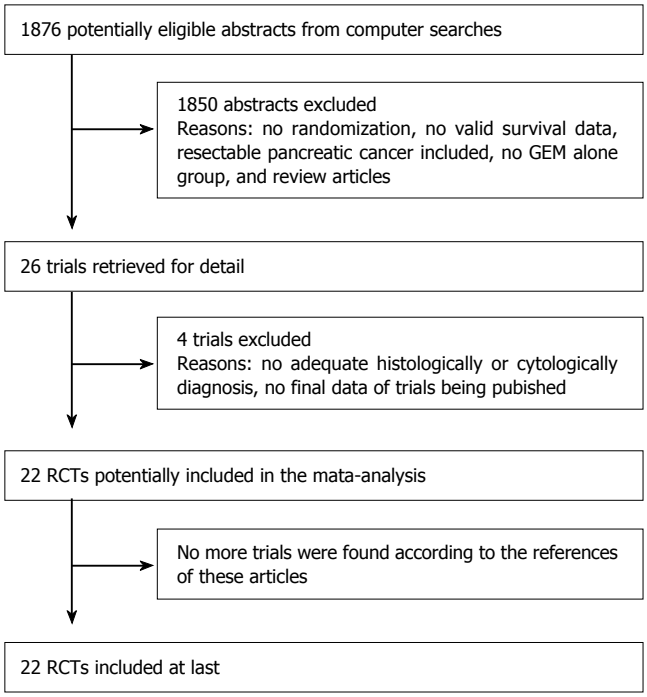

Figure 1 The flow chart. GEM: gemcitabine; RCTs: randomized controlled trials.

RESULTS

Trial flow

The flow chart of our study is shown in Figure 1. Because the trial reported by Degen *et al*[4] involved some patients diagnosed by imageology, we excluded this trial from our analysis. Of the 26 trials, three reported by Ohkawa *et al*[5], Richards *et al*[6], and Shapiro *et al*[7], were excluded because of no final data. Both reviewers finally agreed to include 22 RCTs involving 5473 APCa patients in the meta-analysis.

Characteristics of selected trials

These prospective randomized controlled studies are summarized in Table 1. All selected trials for inclusion strictly according to prior selection criteria, were prospective, randomized and well designed, and the clinical characteristics were matched for age, stage, performance status, and so on. All studies reviewed were considered high in quality, for they achieved a score of 3 or higher in the assessment scale of Jadad's study design. Patients eligible for these studies had histologically or cytologically

Table 1 Randomized controlled trials (GEM combination *vs* GEM alone)

Studies	Intervention	Patients	OS (d)	6-mo survival (%)	1-yr survival (%)	6-mo TTP/PFS/ TTF rate (%)	ORR (CR + PR) %	CBR	Jadad score
Scheithauer 2003[8]	Gem	42	246	59.4	37.2	24.6 (PFS)	6/42	10/30	3
	Gem + Capecitabine	41	285	67.7	31.8	36.9	7/41	15/31	
Colucci 2002[9]	Gem	54	140	31.5	11	18 (TTP)	5/48	21/43	3
	Gem + DDP	53	210	47	11.3	28	14/45	20/38	
Wang XY 2002[10]	Gem	20	-	81.3	31.3	-	1/16	14/16	3
	Gem + DDP	22	-	61.6	11.1	-	2/18	14/20	
Gansauge 2002[11]	Gem	28	144	32	11	-	1/28	-	3
	Gem + NSC-631570	28	279	64	29	-	6/28	-	
Berlin 2002[12]	Gem	162	162	42	15.5	32/160 (PFS)	9/162	-	3
	Gem + 5-FU	160	201	55	21.9	41/158	11/160	-	
Bramhall 2002[13]	Gem + placebo	119	164	43	17	23 (TTF)	14/88	-	5
	Gem + marimastat	120	165.5	47	18	29	11/97	-	
Cutsem 2004[14]	Gem + placebo	347	182	49	24	-	28/347	-	5
		341	193	53	27	-	20/341	-	
Louvet 2005[15]	Gem	156	213	60.4	27.8	27.4 (PFS)	27/156	26.9	3
	Gem[1] + Oxaliplatin	157	270	68	34.7	43	42/157	38.2	
Reilly 2004[16]	Gem	174	186	51	21	27 (TTP)	9/127	-	3
	Gem + DX-8951f	175	201	54	23	39	12/147	-	
Richards 2004[17]	Gem	282	189	50.9	20.1	27.6 (PFS)	20/220	-	3
	Gem + Pemetrexed	283	186	50.9	21.4	32.1	42/230	-	
Li CP 2004[18]	Gem	25	138	20.3	13.6	11.8 (TTP)	3/25	9/25	3
	Gem + DDP	21	168	31.1	6.3	11.8	2/21	6/21	
Reni 2004[19]	Gem	47	-	63.9	21.3	12.9 (PFS)	4/47	5/20	3
	Gem + 5-FU + DDP + EPI	52	-	64.6	38.5	37.4	20/52	15/23	
Viret 2004[20]	Gem	41	201	58.3	25.1	10 (TTF)	2/41	-	3
	Gem + DDP	42	241	55.5	32.4	14	3/42	-	
Rocha Lima 2004[21]	Gem	180	198	52.9	22	21.9 (TTP)	8/180	-	3
	Gem + Irinotecan	180	189	50.7	21	30.6	29/180	-	
Costanzo 2001[22]	Gem	49	217	59	14.5	-	4/48	-	3
	Gem + 5-FU	44	210	59	23.3	-	5/43	-	
Heinemann 2003[23]	Gem	97	180	48.6	22.5	25.6 (TTP)	8/93	-	3
	Gem + DDP	95	228	59.4	27.5	39.3	10/92	-	
Kulke 2004[24]	Gem[2]	45	-	24/45	-	-	-	-	3
	Gem + DDP	45	-	23/45	-	-	-	-	
	Gem + Docetaxel	49	-	22/49	-	-	-	-	
	Gem + Irinotecan	44	-	21/44	-	-	-	-	
Richards 2002[25]	Gem + Placebo	88	213	62.9	20.4	25.9 (TTF)	5/63	-	5
	Gem + CI-994	86	191	60.8	18.5	16.7	1/61	-	
Moore 2005[26]	Gem + Erlotinib	285	191	58	24	32 (PFS)	23/268	-	5
	Gem + placebo	284	177	49	17	25	21/262	-	
Stathopoulos 2005[27]	Gem	70	195	50	21.82	-	7/70	-	3
	Gem + Irinotecan	60	192	60	24.29	-	9/60	-	
Riess 2005[28]	Gem	236	186	53	20	30 (TTP)	17/236	-	3
	Gem + 5-FU/CF	230	175.5	49	20	29	11/230	-	
Herrmann 2005[29]	Gem	157	219	62	27	42 (PFS)	12/152[3]	-	3
	Gem + Capecitabine	159	252	60	31	42	15/148	-	

[1]Gemcitabine 1 g/m^2 as a 100-min infusion; [2]Gemcitabine 1500 mg/m^2 at a fixed dose rate of 10 mg/m^2 per minute; [3]RECIST criteria.

proved pancreatic cancer, with same baseline data and without evidence of selection bias. Of the 22 trials, seven were randomized phase Ⅱ trials, and the others were randomized phase Ⅲ trials. The 6-mo survival rate was extracted from each of the 22 trials, and objective remission rates were recorded in most of the trials. Only a few trials provided CBR, PFS, TTP and TTF (time of treatment failure).

Overall survival

The 5473 randomized patients from 22 RCTs, 2772 in the GEM combination group and 2701 in the GEM alone group, were included in the meta-analysis. The result of the test for heterogeneity of the therapeutic effect was not significant ($P = 0.19$). Therefore, we selected a fixed effect model. There was a significant improvement in 4% of the GEM combination group in 6-mo survival rate (95% CI 0.01-0.06, $P = 0.008$). The results of the meta-analysis in 6-mo survival rate are presented in Figure 2.

With the same technique, 5292 patients from 21 RCTs were analyzed. In the GEM combination group, a 3% improvement was made in 1-year survival rate as compared with the GEM alone group, and this difference being statistically significant (95% CI 0.01-0.05, $P = 0.01$).

The 4912 randomized patients from 21 RCTs, 2461 in the GEM combination group and 2451 in the GEM alone group, were included in the meta-analysis. The result of the test for heterogeneity of the therapeutic effect

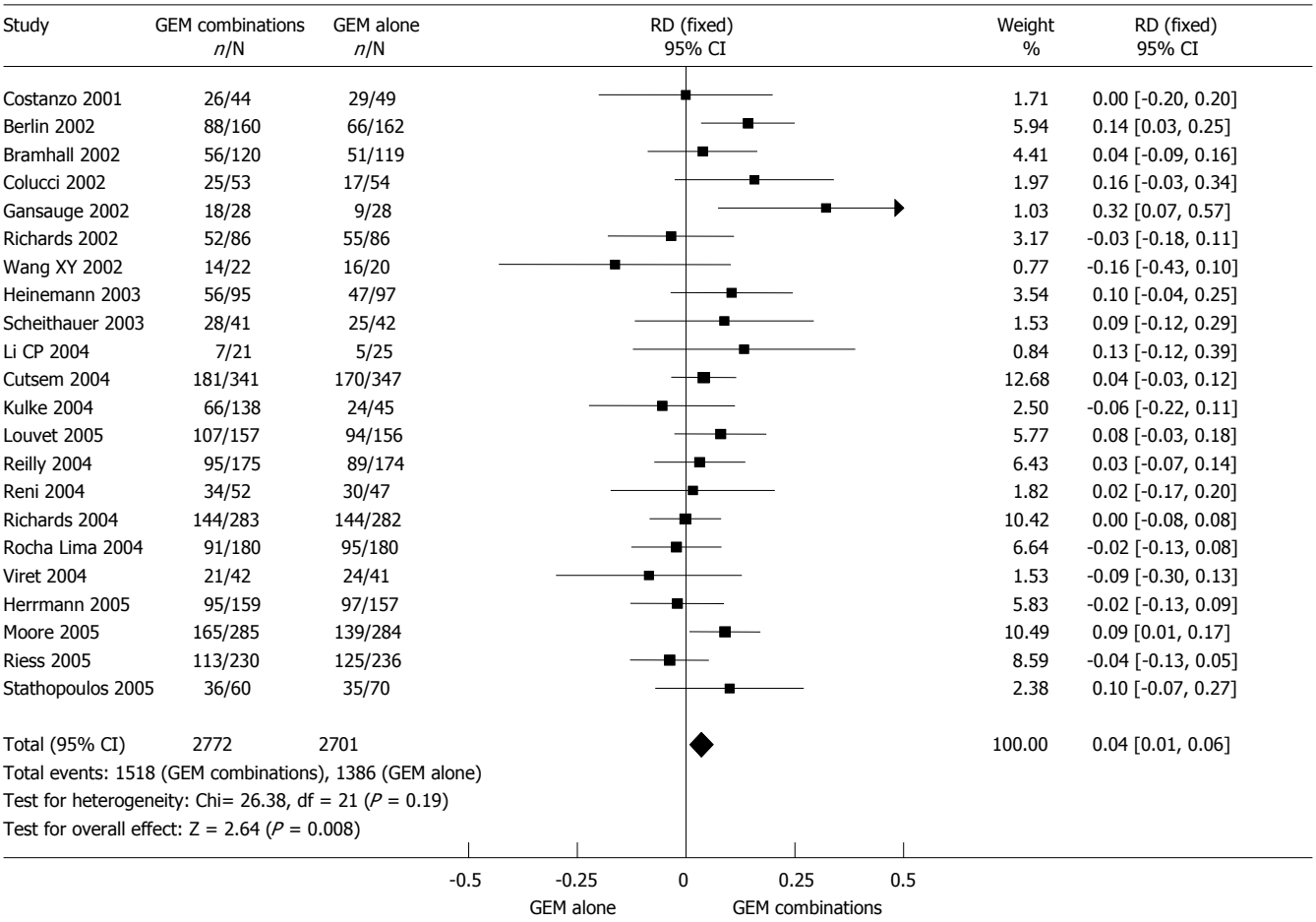

Figure 2 Fixed effect model on RD of 6-mo survival rate.

was significant ($P < 0.0001$). A random effect model was adopted. There was a significant improvement in 4% of the GEM combination group in ORR (95% CI 0.01-0.07, $P = 0.02$). The outcome of the meta-analysis in ORR is presented in Figure 3.

The 580 randomized patients from 6 RCTs, 290 in the GEM combination group and 290 in the GEM alone group, were included in the meta-analysis. The result of the test for heterogeneity of the therapeutic effect was not significant ($P = 0.05$).

A fixed effect model was used. There was a significant improvement in 10% of the GEM combination group in CBR (95% CI 0.02-0.17, $P = 0.01$). The outcome of the meta-analysis in CBR is shown in Figure 4.

Six-month TTP/PFS rate

TTP/PFS was defined as the period from randomization to documented disease progression for TTP or to disease progression or death for PFS. In almost all of the trials, patients recruited with good performance status died of disease progression, so TTP was very close to PFS. Therefore, we can analyze TTP and PFS together.

The 3783 randomized patients from 13 RCTs, 1889 in the GEM combination group and 1894 in the GEM alone group, were included in the meta-analysis. The result of the test for heterogeneity of the therapeutic effect was not significant ($P = 0.20$). A fixed effect model was used. Significant improvement was found in 7% of GEM combination group in 6-mo TTP/PFS rate (95% CI 0.04-0.10, $P < 0.00001$). The meta-analysis in TTP/PFS is presented in Figure 5.

Toxic effects of chemotherapy

Toxic effects of 21 RCTs are summarized in Table 2 (only Grade 3-4 toxic effects were recorded). Main toxic effects were analyzed. Grade 3-4 toxicity was higher in GEM combination group for neutropenia (RD = 5%, 95% CI 0.01-0.10, $P = 0.02$), thrombocytopenia (RD = 5%, 95% CI 0.02-0.08, $P = 0.002$) and vomiting/nausea (RD = 3%, 95% CI 0.00-0.05, $P = 0.02$), all reached significant difference.

Assessment for publication bias

Figures 6 and 7 represent funnel plots that test for publication bias. Funnel plots for the 6-mo survival rate (Figure 6) and 1-year survival rate (Figure 7) supported the lack of evidence for publication bias.

Subgroup analysis

Table 3 shows the subgroup analyses in 6-mo survival rate. It revealed that only the combined chemotherapy consisting of GEM plus a new targeted drug yielded a 6% higher survival rate as compared with chemotherapy of GEM alone.

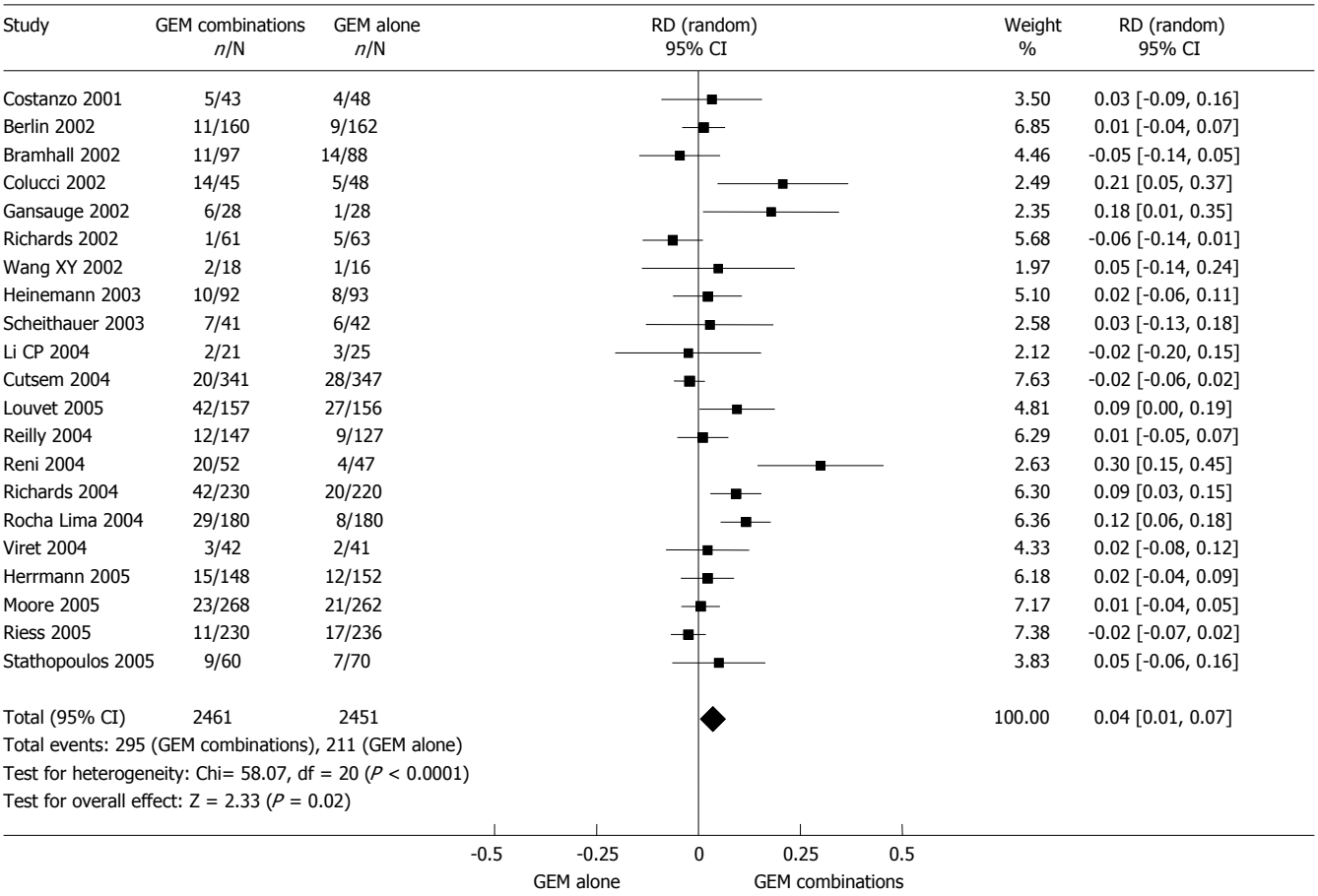

Figure 3 Random effect model on RD of ORR.

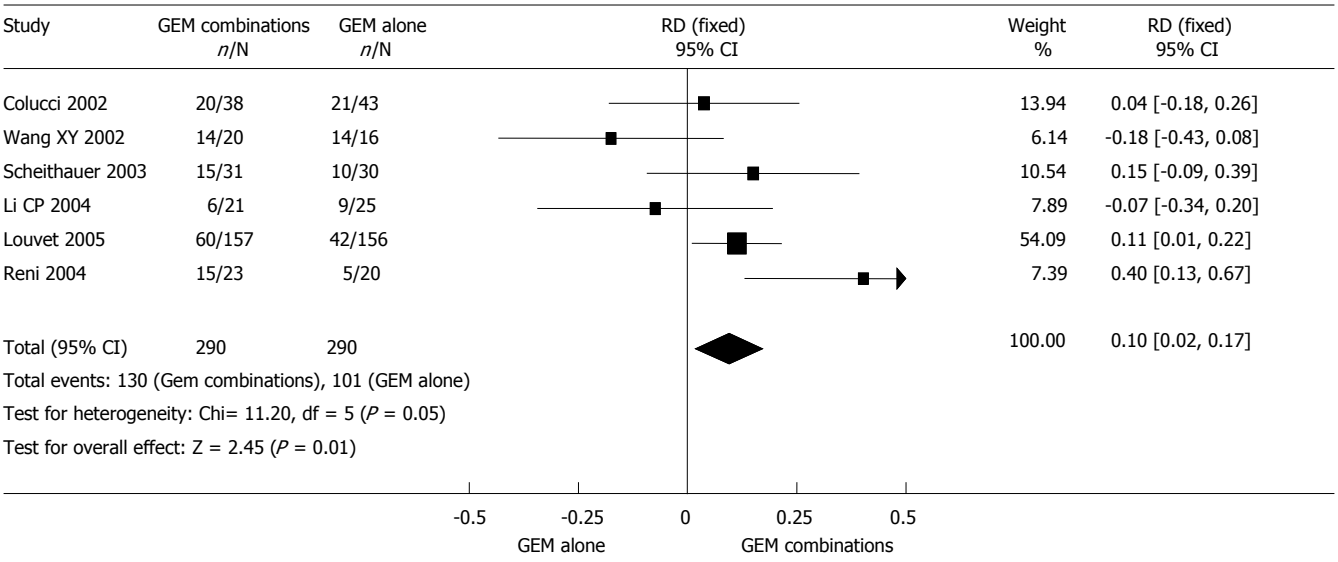

Figure 4 Fixed effect model on RD of CBR.

DISCUSSION

To improve the clinical results of GEM, Phase Ⅱ-Ⅲ trials have been made recently to evaluate the efficacy of combination of GEM with other drugs which were shown to be synergistic *in vitro*, such as 5-fluorouracil (5-FU), DDP, topotecan, *etc*[30,31]. Many trials demonstrated that combined GEM chemotherapy improved ORR and PFS compared with GEM alone, and a few trials reported significant OS advantage (Table 1).

The present meta-analysis shows that GEM combination produced a significant survival advantage as compared with GEM alone in patients with APCa. GEM combination was also found superior to GEM alone in

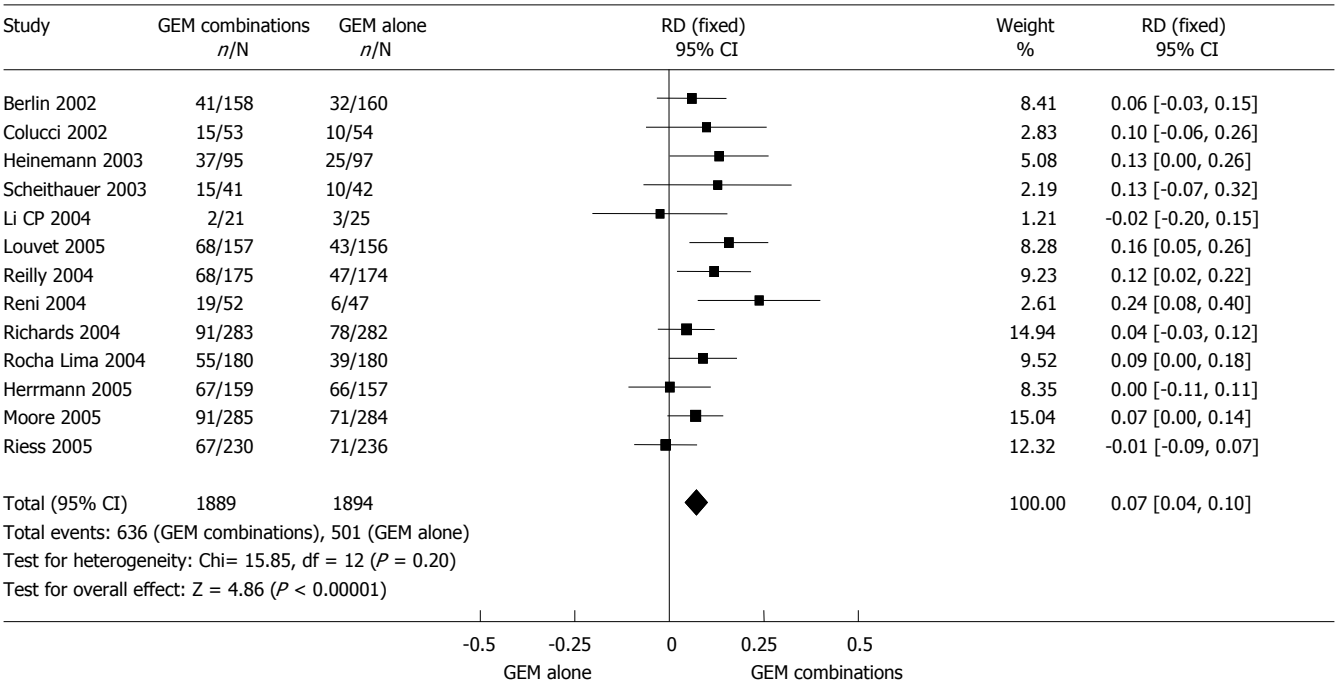

Figure 5 Fixed effect model on RD of 6-mo TTP/PFS rate.

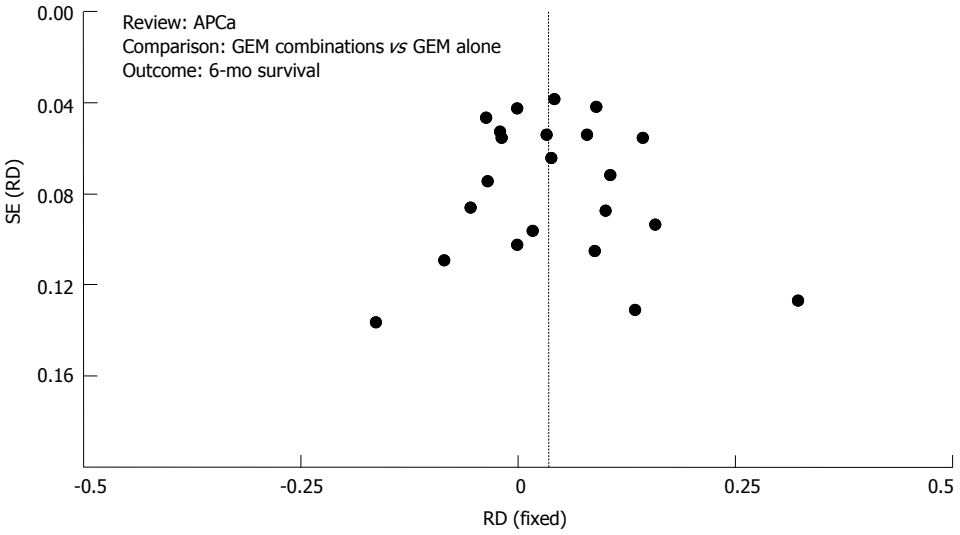

Figure 6 Funnel plots for 6-mo survival rate.

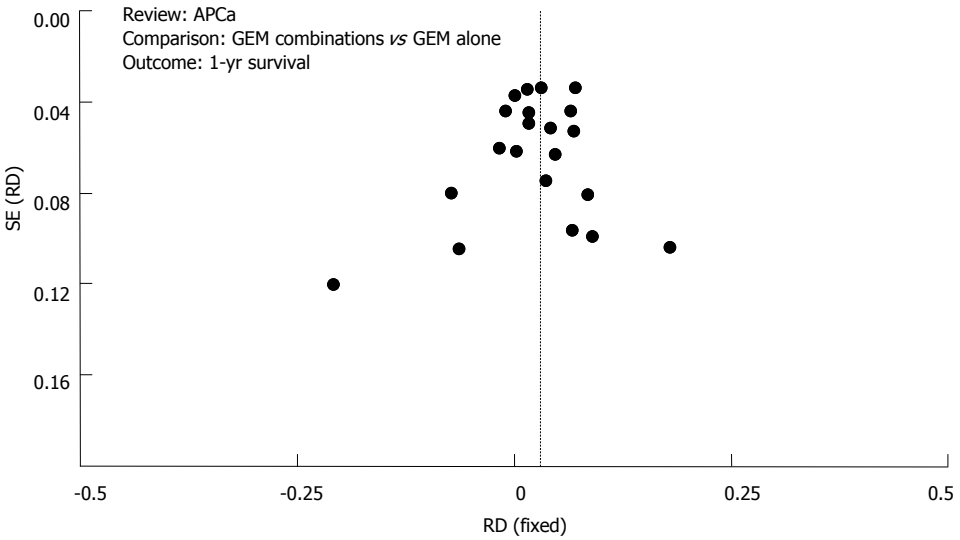

Figure 7 Funnel plots for 1-yr survival rate.

Table 2 Toxic effects recorded from randomized controlled trials (Grade 3-4 toxic effects)

Studies	Intervention	Neutrophils	Platelets	Anemia	Infection	Nausea/vomit	Mucositis	Diarrhea
Scheithauer2003[8]	Gem	3/39	1/39	0	0	0	0	0
	Gem + Capecitabine	4/40	0	2/40	0	0	0	2/40
Colucci 2002[9]	Gem	5/53	1/53	2/53	-	1/53	1/53	0
	Gem + DDP	9/51	1/51	3/51	-	1/51	0	2/51
Wang XY 2002[10]	Gem	5/19	7/19	2/19	-	0	-	-
	Gem + DDP	7/21	8/21	9/21	-	2/21	-	-
Gansauge2002[11]	Gem	-	-	-	-	3/28	-	1/28
	Gem + NSC-631570	-	-	-	-	1/18	-	0
Berlin 2002[12]	Gem	8/158	17/158	16/158	-	30/158	3/158	/158
	Gem + 5-FU	11/158	30/158	16/158	-	29/158	2/158	/158
Bramhall 2002[13]	Gem + placebo	9/119	-	7/119	12/119	16/119	-	-
	Gem + marimastat	3/120	-	3/120	11/120	13/120	-	-
Cutsem 2004[14]	Gem + placebo	103/342	41/342	55/342	-	58/342	-	10/342
	Gem + R115777	132/331	50/331	66/331	-	46/331	-	13/331
Louvet 2005[15]	Gem	2/156	5/156	16/156	-	12/156	-	2/156
	Gem + Oxaliplatin	2/157	22/157	10/157	-	30/157	-	9/157
Reilly 2004[16]	Gem	24/157	7/157	13/157	-	17/157	-	2/157
	Gem + DX-8951f	50/168	28/168	11/168	-	33/168	-	6/168
Richards 2004[17]	Gem	35/273	17/273	8/273	-	18/273	-	2/273
	Gem + Pemetrexed	123/273	49/273	38/273	-	18/273	-	8/273
Li CP 2004[18]	Gem	2/25	1/25	2/25	-	-	-	-
	Gem + DDP	4/21	5/21	2/21	-	-	-	-
Reni 2004[19]	Gem	9/47	1/47	2/47	-	4/47	1/47	-
	Gem + 5-FU + DDP + EPI	22/52	15/52	2/52	-	3/52	2/52	-
Viret 2004[20]	Gem	16/40	5/40	11/40	-	3/40	-	-
	Gem + DDP	23/41	14/41	14/41	-	9/41	-	-
Rocha Lima 2004[21]	Gem	54/169	24/169	22/169	-	31/169	-	3/169
	Gem + Irinotecan	65/173	34/173	27/173	-	53/173	-	33/173
Costanzo 2001[22]	Gem	1/49	0	3/49	-	0	0	0
	Gem + 5-FU	1/41	1/41	3/41	-	1/41	2/41	0
Heinemann 2003[23]	Gem	8/97	10/97	10/97	2/97	6/97	2/97	4/97
	Gem + DDP	10/95	4/95	13/95	1/95	21/95	4/95	3/95
Kulke 2004[24]	Gem	27/58	15/58	6/58	6/58	13/58	-	1/58
	Gem + DDP	29/62	27/62	11/62	2/62	24/62	-	0/62
	Gem + Docetaxel	19/65	7/65	8/65	8/65	10/65	-	5/65
	Gem + Irinotecan	12/60	9/60	4/60	4/60	17/60	-	10/60
Moore2005[26]	Gem + Erlotinib	71/282	28/282	34/282	45/282	20/282	<1	17/282
	Gem + placebo	73/280	34/280	34/280	39/280	20/280	0	6/280
Stathopoulos 2005[27]	Gem	8/70	0/70	2/70	0	1/70	0	2/70
	Gem + Irinotecan	10/60	2/60	2/60	0	1/60	0	2/60
Riess 2005[28]	Gem	27/225	15/225	15/225	19/225	16/225	-	9/225
	Gem + 5-FU/CF	26/220	28/220	18/220	12/220	30/220	-	8/220
Herrmann 2005[29]	Gem	30/153	7/153	9/153	-	5/153	1/153	3/153
	Gem + Capecitabine	34/155	8/155	9/155	-	8/155	0/155	8/155

5-FU: 5-fluororacil; EPI: Epirubicin.

terms of ORR, CBR and 6-mo TTP/PFS. Although most of the selected RCTs showed no significant survival advantage in the GEM combination group, many trials demonstrated slight survival benefit. Physicians should carefully interpret these results when they apply them in clinical practice because GEM combined with other regimens might lead to reversed therapeutic effects.

Straightforward conclusions from the results of this meta-analysis do support the use of GEM combination in patients with APCa, but toxicities from intensive chemotherapy may obliterate the survival benefit of GEM combination. In another meta-analysis, we had reported that the regimens GEM plus DDP were not superior to GEM alone in patients with APCa, which produced more side effects[32]. Furthermore, the subgroup analyses did not show any significant survival advantage in most of GEM combination groups, such as GEM plus 5-FU, GEM plus topoisomerase I inhibitor, and so on. It indicates that not all GEM combined chemotherapy have therapeutic advantage. We suggest that GEM combination, including GEM plus oxaliplatin, and GEM plus erlotinib, should be considered as optimal treatment for patients with APCa. In addition, we found that patients with good perforemance status gained great survival advantage in the sub-group analyses as reported by many other authors[28,29,12]. In our opinion, GEM combination should be applied to patients with good performance status, but carefully to the weak patients.

We found that patients receiving GEM-based combination therapy developed side effects more frequently, including neutropenia, thrombocytopenia and vomiting/nausea , which might lead to a deterioration in

Table 3 Subgroup analyses on 6-mo survival rate

Subgroups	Trials	Patients	Mode	RD [95% CI]	*P*
GEM plus targeted drug *vs* GEM alone	[13, 14, 26]	1496	Fixed	0.06 [0.01, 0.11]	0.02
GEM plus DDP *vs* GEM alone	[9, 10, 18, 20, 23, 24]	560	Fixed	0.05 [-0.03, 0.13]	0.24
GEM plus 5-FU *vs* GEM alone	[12, 22, 28]	881	Random	0.04 [-0.09, 0.17]	0.57
GEM plus topoisomerase I inhibitor *vs* GEM alone	[16, 21, 24, 27]	928	Fixed	0.01 [-0.05, 0.08]	0.72
GEM plus capecitabine *vs* GEM alone	[8, 29]	399	Fixed	0.00 [-0.08, 0.10]	0.97

quality of life (QOL). However, the significant advantage of CBR and TTP/PFS in the GEM combination might be converted to the improvement of QOL**.** Because the primary role of chemotherapy in patients with APCa is palliative, the influence on the QOL of the patients is an important issue in determining the true value of the therapy. However, because the methods for QOL assessment from the included trials were quite different, there was no valid meta-analysis of QOL . We also noted that the CBR analysis was made in only six trials, so the result was still unreliable.

The mata-analysis was based on RCTs with high quality. We carried out a comprehensive search of the literature with barely all of cancer database. Publication bias is frequently cited as a reason for lack of validity in meta-analyses. It could occur if studies finding no association between exposure and disease were less likely to be submitted and accepted for publication than studies finding a positive association. In fact, the results of most of the studies in our meta-analyses were negative, as stated by the authors. The funnel plots also showed no evidence of publication bias. Therefore, our meta-analysis provided a valid assessment and creditable results.

Several technical issues have to be mentioned regarding this meta-analysis. One major limitation is the data source extracted from abstracted data and not individual patient data (IPD). In general, an IPD-based meta-analysis would give a more robust estimation for the association, therefore, we should interpret the results with care, especially for a positive result. Clearly, further investigations using IPD should be conducted to examine the main end points. Publication bias is a significant threat to the validity of meta-analysis. Although we detected no evidence of publication bias using the graphical method, it is difficult to completely rule out this possibility. Heterogeneity among trials can be another limitation of our meta-analysis. Although we applied a random-effect model that takes possible heterogeneity into consideration, there were still many factors causing heterogeneity, such as different drug combination, two infusion methods of gemcitabine and so on.

In conclusion, the meta-analysis indicates that GEM-based combination therapy may improve the overall survival and palliation in optimal patients with APCa as compared with GEM alone. Although the application of GEM combination is still controversial, it is a progressive method from the prospective view of point. At the same time, new regimens of drug administration should be explored in future studies.

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CLINICAL RESEARCH

Relationship between antral distension and postprandial symptoms in functional dyspepsia

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Abstract

AIM: To investigate in patients with functional dyspepsia (FD) after an every-day meal whether (1) gastrointestinal (GI) and extra-GI symptoms had any relation with the degree of antral volume, (2) the onset of postprandial symptoms was associated with, and may predict, delayed gastric emptying.

METHODS: In 94 symptomatic FD patients, antral volume variations and gastric emptying were assessed with ultrasonography after a 1050 kcal meal. Symptoms were evaluated with a standardized questionnaire. The association of GI and extra-GI symptoms with antral volumes and gastric emptying were estimated with logistic regression analysis.

RESULTS: Forty percent of patients did not report any symptoms after a meal. Compared to the healthy controls, the antrum was more distended in patients throughout the entire observation period and 37 (39.4%) patients had delayed gastric emptying. Only postprandial drowsiness was associated with antral volume variations (AOR = 1.42; $P < 0.001$) and with delayed gastric emptying (AOR = 3.59; $P < 0.03$).

CONCLUSION: In FD patients, GI symptoms are neither associated with antral distension nor with gastric emptying. Drowsiness is associated with antral distension and delayed gastric emptying. The onset of drowsiness is preceded by an increment of antral distension and the duration of the symptom appears to be related to the persistence of antral distension.

Key words: Antral distension; Functional dyspepsia; Gastric emptying, Ultrasonography

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INTRODUCTION

Patients with functional dyspepsia (FD)[1] complain of several gastrointestinal (GI) and extra-gastrointestinal (extra-GI) symptoms[2-5] that are usually associated with food ingestion[6]. Several pathophysiological abnormalities have been implicated in the etiology of symptoms, but so far they failed to establish a clear-cut association between symptoms and specific function abnormalities and often similar studies provided contrasting findings[6-13]. Despite symptoms are often related to food ingestion, few studies have assessed the relationship between the occurrence of GI symptoms after meal ingestion and gastric functions[6,10,11,14-19] and reported non-univocal results. In addition, investigations performed while patients are symptom-free may miss their underlying pathophysiological mechanisms because as the symptoms, they may wax and wane over time[20]. Ricci *et al*[14] firstly and others subsequently[15,16,18,21,22] reported a distended fasting antrum and an increased post-prandial antral volume in patients with FD as compared with healthy controls, suggesting that an impaired motor function of the distal stomach is present in a subgroup of patients with FD. However, only two studies[14,15] evaluated the temporal association between the onset of postprandial GI symptoms and antral distension assessed with ultrasonography (US). The first study reported a close association between the onset of the postprandial GI complaints[14] and antral volume increase in the majority (71%) of patients. The second study reported an association between bloating and increased antral area[15]. We have recently shown that a subgroup of patients with FD reported postprandial drowsiness as a bothersome symptom, in addition to the GI dyspeptic ones[5]. Drowsiness is a subjective experience often reported after food ingestion[23]. It has also been shown that solid but not liquid meal results in a decreased sleep onset latency in healthy volunteers[24], suggesting that a post-ingestion mechanism rather than a cephalic stimulus is involved as an initial trigger in mediating the phenomenon.

We aimed to further evaluate the relationship between the onset of postprandial symptoms, both GI and extra-GI symptoms, such as headache and drowsiness, and the modality of the postprandial antral volume increase in FD patients.

The primary aim of the present study was to investigate with regression analysis models, in controlled condition, after an every-day balanced meal, whether upper GI and extra-GI symptoms had any relation with the degree of antral volume assessed with US in FD patients. The secondary aim was to evaluate whether the onset of postprandial symptoms was associated to, and may predict, delayed gastric emptying.

MATERIALS AND METHODS

Subjects

Two hundred and seventeen consecutive patients with chronic symptoms of dyspepsia (143 females, 74 males; age, 42.4 ± 12.2 years;) referred to the gastroenterology outpatient clinic were prospectively assessed. Functional dyspepsia (FD) was defined according to the Rome Ⅱ criteria[1]. Organic abnormalities, psychiatric illnesses, eating disorders, history of alcohol and caffeine abuse, use of NSAID, steroids or drugs affecting gastric function, previous GI surgery (except appendectomy and cholecystectomy) and systemic disorders were ruled out by history, clinical examination, biochemical investigations, upper GI endoscopy, and transabdominal US. Dyspeptic patients with Rome diagnostic criteria of irritable bowel syndrome (IBS) and/or referring heartburn and/or regurgitation as predominant or frequent symptoms were excluded from the study.

Severity of epigastric pain and upper abdominal discomfort was graded 0-4 according to its effect on patient's daily activities: 0 = absent; 1 = mild (present but easily bearable if distracted by usual activities); 2 = moderate (bearable but not influencing usual activities); 3 = relevant (influencing usual activities); and 4 = severe (interruption of usual activities)[5]. Patients were symptomatic at the time of, and in the 3 weeks preceding, the investigation. Dyspeptic symptoms had to be present more than 3 days a week, with pain and discomfort scored at least as moderate (≥ 2).

Overall 123 patients (80 females, 43 males; age, 44 ± 13.5 years) were excluded from the study because of the following diagnosis: 59 with gastroesophageal reflux disease; 15 with IBS; 14 with peptic ulcer disease; 16 with migraine; 3 with psychiatric disorders; 2 with celiac disease; and 14 with not properly reporting symptoms during the test.

Ninety-four consecutive patients (63 females, 31 males; age, 42 ± 12 years) fulfilling Rome Ⅱ diagnostic criteria of functional dyspepsia[1] and 21 healthy subjects (13 females, 8 males; age, 30 ± 8.5 years) without GI symptoms participated in the study.

Informed consent was obtained from each subject and the Local Ethics Committee approved the study protocol.

Assessment of symptoms

An experienced gastroenterologist (EC) interviewed the patients before the study. GI symptoms were enquired by means of the validated Italian version of the Rome Ⅱ modular questionnaire[25]. The validation process and validity of the translated questionnaire have been formally assessed and approved by the Coordinating Committee of the Rome Foundation (on files of the Committee). The questionnaire also includes items inquiring on demography (5 items), daily habits (10 items), meal timing and composition, alcohol consumption, smoking and sleep patterns, past medical history (3 items), somatic extra-GI symptoms (5 items) as previously reported[2].

Frequency and time relationship with meal ingestion were assessed for each of the following dyspeptic symptoms, as defined by the Rome Ⅱ criteria[1]: pain or discomfort centered in the upper abdomen, nausea, vomiting, fullness, bloating, early satiety, epigastric burning and belching. Postprandial drowsiness was defined as a state of impaired awareness associated with a desire or inclination to sleep[26]. Furthermore, patients were requested to refer any other symptom they considered to be bothersome and related to meal ingestion.

Study protocol

Gastric antral volume was evaluated at US as previously described[27,28] with 3.5-MHz convex probe (Tosbee, Toshiba, Japan). Gastric emptying was evaluated with US according to previously validated and standardized methods[27-32]. All drugs affecting the GI tract were discontinued at least 3 d before the study. Subjects refrained from smoking 12 h before and during the examination. Beverages, including water, coffee and tea were not allowed before and during the examination. The presence of sleep disturbances the night before the test and the quality of sleep in the last month were specifically enquired. After an overnight fast, subjects ate an ordinary standard solid meal of 1050 kcal containing 140 g bread, 70 g cheese, 80 g ham, (50 % carbohydrates, 25% lipids, 25% proteins) and 250 mL of water. The time of meal ingestion did not exceed 30 min (range, 15-30 min). The same proven-skilled operator (NP) performed gastric antral US measurements, with the subjects standing in the upright position. Subjects were studied in fasting condition, soon after the meal ingestion, at 30 and 60 min after the end of the meal ingestion, and at 60 min intervals thereafter for 300 min. In the intervals between measurements subjects could move freely.

Delayed gastric emptying was defined as the final antral volume exceeding the mean value plus 2SDs of the healthy controls[5].

Patients and healthy controls were requested to report every 30 min any symptom occurring after the meal ingestion. The ultrasonographer was blind for the referred historical symptoms.

Statistical analysis

To compare patients and healthy controls as well as temporal meal-related antral volume variations, arithmetic mean, standard deviation, median values and interquartile ranges were calculated. Continuous variables (age, body mass) were compared using student's t-test. Box-plots[33] were used to provide an immediate graphical evaluation of

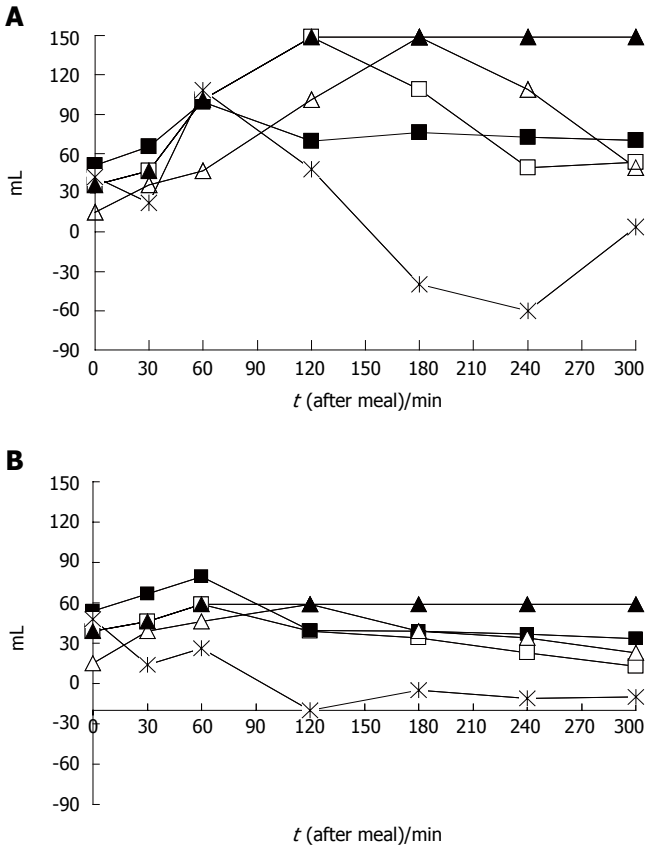

Figure 1 Antral volume measurements and their transformations. **A**: Patients referring postprandial drowsiness during the study period; **B**: Patients not referring symptoms during the study period. Open quadrangle: current antral volume; asterisk: antral volume delta variation between two consecutive measurements; Closed quadrangle: mean weighted antral volume; Closed triangle: maximal antral volume; Open triangle: antral volume value at the preceding time intervals.

the postprandial gastric antral volume distribution during the study period. Linear regression analysis for repeated measurements was applied to evaluate during the study period whether there were differences in the antral volumes between patients and healthy controls. The cumulative probability of developing symptoms during the 5 h study period was evaluated using the Kaplan-Meier method. Logistic regression models[34] were applied to estimate crude (OR) and adjusted odds ratios (AOR) for age, body mass index (BMI) and gender, and 95% confidence intervals (95%CI) of having an association between antral volume variations and presence/absence of each GI and extra-GI symptom at each time interval evaluated. We also investigated specific transformations of the current gastric antral volume evaluated for a 10 mL unit change as follows (Figure 1 A and B): (1) the antral volume value at the preceding time interval; (2) the antral volume delta variation between two consecutive measurements; (3) the mean weighted antral volume value at each measurement (i.e., at each time when the volume was measured, the value obtained as the arithmetic mean of the current value together with all previous measurements); (4) the maximal antral volume value reached after a meal between the current and the previous measurement.

Logistic regression analysis was also applied to estimate crude and adjusted odds ratios of having delayed gastric emptying for each symptom, fasting antral volume, gender, age, and BMI. We reported results from multiple logistic regression analyses, obtained through a backward selection strategy having excluded factors with a P value >0.20 obtained by a likelihood-ratio test[33]. Two-sided P values were defined statistically significant when $P < 0.05$, and marginally significant when $0.05 < P < 0.2$. All the analyses were performed using STATA release 8.0[35].

Table 1 Major demographic characteristics of investigated subjects

	Healthy controls (n = 21)	FD patients (n = 94)	P
Age median (IQR) yr	27 (26-29)	42 (31-52)	< 0.001
BMI median (IQR) kg/m^2	22.4 (20-23)	22.0 (19.8-25.0)	0.47
Female (%)	13 (61.9%)	62 (66.0%)	0.72

IQR: Interquartile range.

RESULTS

Study population and GI and extra-GI symptoms profile

At inclusion, all FD patients reported frequent pain or discomfort (60% of them almost daily) graded at least as moderate. Demographic characteristics of the subjects evaluated are summarized in Table 1. There was no significant difference between the healthy controls and the FD patients for BMI and gender, whereas the controls were younger ($P < 0.001$) than the FD patients. After a meal, none of the healthy subjects reported any symptoms. Of the FD patients, 39 (41.5%) did not refer any symptoms and 55 (58.5%) reported one or more GI and extra-GI symptoms. In this group of 55 symptomatic patients, postprandial drowsiness was reported as a predominantly bothersome symptom in 17 patients, presenting as the only symptom in 3, in association with pain in 3, with fullness in 6, with pain and fullness in 2, with belching in 2 and with bloating in 1. Reported symptoms' severity in the medical history did not significantly differ between the patients with and without postprandial symptoms during the investigation. The cumulative probability to develop at least one of the GI and extra-GI symptoms was 58.5% (Figure 2). None of the patients referred early satiety, vomiting and heartburn (not shown in Figure 2). Despite the patients with history of regurgitation were excluded from the study, 2 patients referred postprandial acid regurgitation (not shown in Figure 2 and Tables 2 and 3). The percentage of patients with symptoms at each evaluated time interval is reported in Table 2. Among the patients who referred at least one symptom, 55.5% had the first symptom within 60 min after meal ingestion. Postprandial drowsiness started later (71.5 ± 64.8 min) than dyspeptic symptoms (38.3 ± 55.4 min, $P < 0.001$).

Antral volume assessments and gastric emptying

Antral volume before, immediately (time 0) and during the 300 min observation period after meal ingestion in

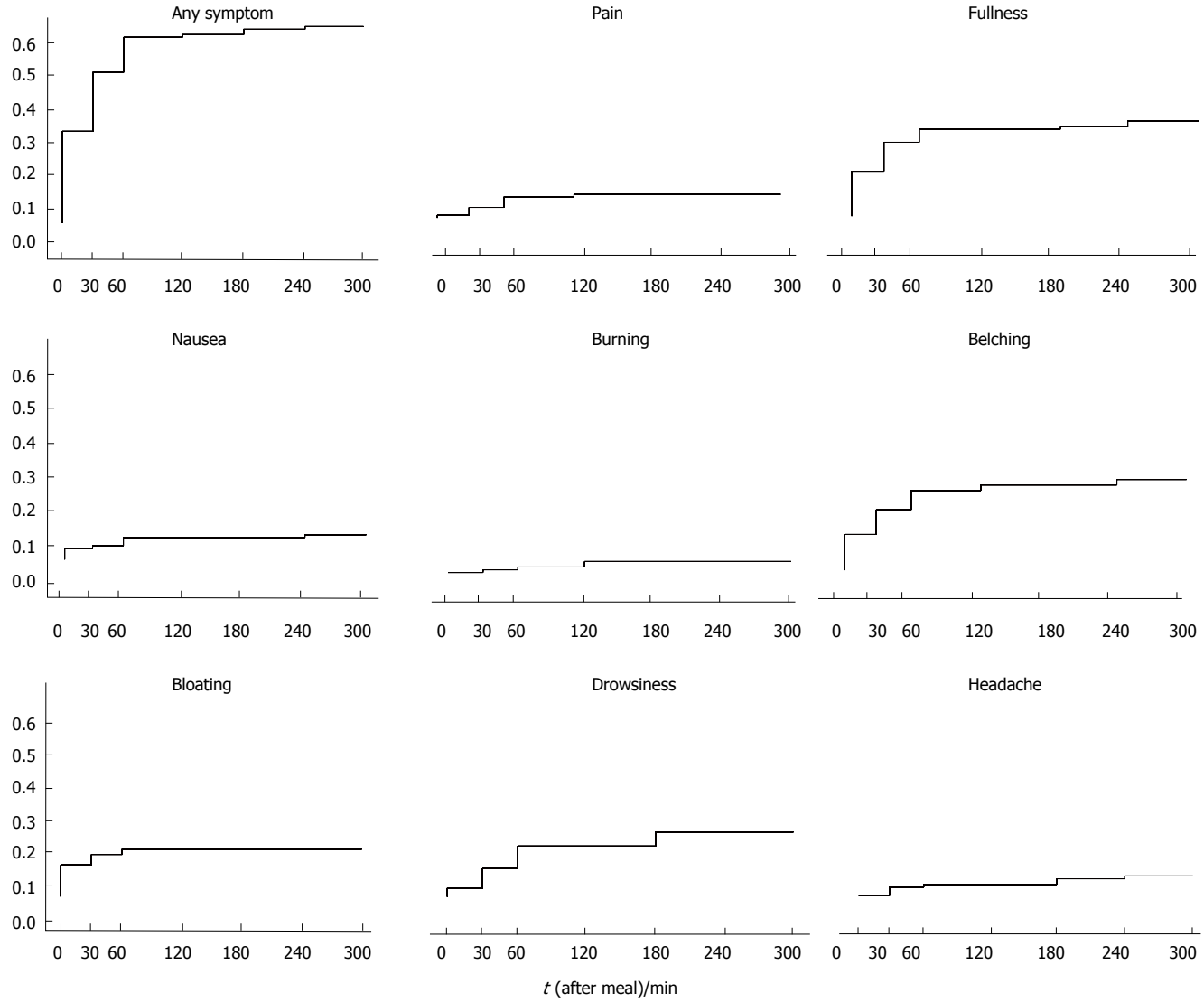

Figure 2 Kaplan-Meier survival curves showing the cumulative probability to develop the evaluated symptoms.

Table 2 Percentage of FD patients with symptoms at each time interval evaluated

Time (min)	Pain (%)	Fullness (%)	Bloating (%)	Nausea (%)	Belching (%)	Burning (%)	Headache (%)	Drowsiness (%)	Any (%)
0	1	13.8	9.6	3.2	10.6	0	0	2.1	26.6
30	3.2	22.3	12.8	4.3	18	1	2.1	7.5	44.7
60	6.4	24.5	14.9	5.3	22.3	2.1	3.2	13.8	54.3
120	6.4	24.5	14.9	5.3	24.5	3.2	3.2	13.8	54.3
180	4.3	20.2	12.8	5.3	20.2	3.2	3.2	17	48.9
240	3.2	14.9	10.6	5.3	18	3.2	4.3	15.9	43.6
300	2	12.8	9.6	5.3	15.9	2.1	2.1	12.8	38.3
At least once	7.4	28.7	14.9	7.4	26.6	3.2	5.3	18	58.5

the controls and the FD patients is shown in Figure 3. In the FD patients, the postprandial antral volume was significantly larger compared to the controls ($P < 0.05$) from the end of meal ingestion up to the final observation. The multiple linear regression model showed that in the postprandial period antral volume, adjusted for gender and age, was higher in the dyspeptic patients compared to the controls with a difference in volume value of 11 mL during the entire period ($P < 0.01$). Gastric emptying was delayed in 37 (39.4%) of the FD patients.

Predictors of postprandial symptoms and gastric emptying in functional dyspepsia

Odds ratios simultaneously adjusted for minutes after meal, BMI, age and gender in predicting the occurrence of GI and extra-GI symptoms evaluated for each of

Table 3 AOR with 95% CI of having GI and extra-GI symptoms on the basis of antral volume variations

	Current AV (per 10 mL unit increase)			Previous AV (per 10 mL unit increase)			Delta AV (per 10 mL unit increase)			Mean weighted AV (per 10 mL unit increase)			Maximal AV (per 10 mL unit increase)		
Symptom	AOR	95% CI	*P*	AOR	95% CI	*P*	AOR	95% CI	*P*	AOR	95% CI	*P*	AOR	95%CI	*P*
Pain	0.96	0.79-1.18	0.72	0.96	0.78-1.17	0.67	1.03	0.93-1.14	0.54	0.94	0.67-1.31	0.72	0.9	0.69-1.19	0.49
Fullness	0.99	0.84-1.17	0.97	0.98	0.82-1.18	0.88	1	0.95-1.06	0.82	0.95	0.73-1.25	0.74	0.97	0.83-1.13	0.68
Bloating	1.21	0.98-1.49	0.07	1.22	0.98-1.51	0.06	0.99	0.93-1.05	0.7	1.37	0.99-1.89	0.05	1.21	0.99-1.48	0.06
Nausea	0.99	0.76-1.3	0.98	1.06	0.8-1.4	0.67	0.84	0.7-1	0.05	1.18	0.82-1.69	0.37	1.17	0.94-1.44	0.15
Burning	0.87	0.55-1.38	0.57	0.81	0.39-1.67	0.58	1.1	0.87-1.39	0.39	0.68	0.2-2.25	0.53	0.64	0.17-2.33	0.5
Belching	1	0.84-1.19	0.95	0.96	0.78-1.17	0.67	1.07	1.01-1.12	0.008	0.94	0.69-1.27	0.69	0.98	0.82-1.17	0.81
Headache	1.04	0.77-1.4	0.79	1	0.77-1.31	0.96	1.03	0.88-1.2	0.69	0.9	0.58-1.43	0.68	0.93	0.7-1.23	0.64
Drowsiness	1.42	1.16-1.73	0.001	1.33	1.08-1.65	0.007	1.12	1.06-1.19	0.001	1.46	1.05-2.01	0.02	1.26	1.03-1.53	0.02
Palpitation	1	0.72-1.4	0.96	0.99	0.71-1.38	0.97	0.98	0.9-1.06	0.67	0.89	0.49-1.62	0.7	0.93	0.65-1.32	0.68
Any symptom	1.13	0.97 -1.31	0.12	1.1	0.94 -1.3	0.24	1.03	0.99 -1.08	0.11	1.15	0.9-1.46	0.25	1.11	0.95-1.29	0.18

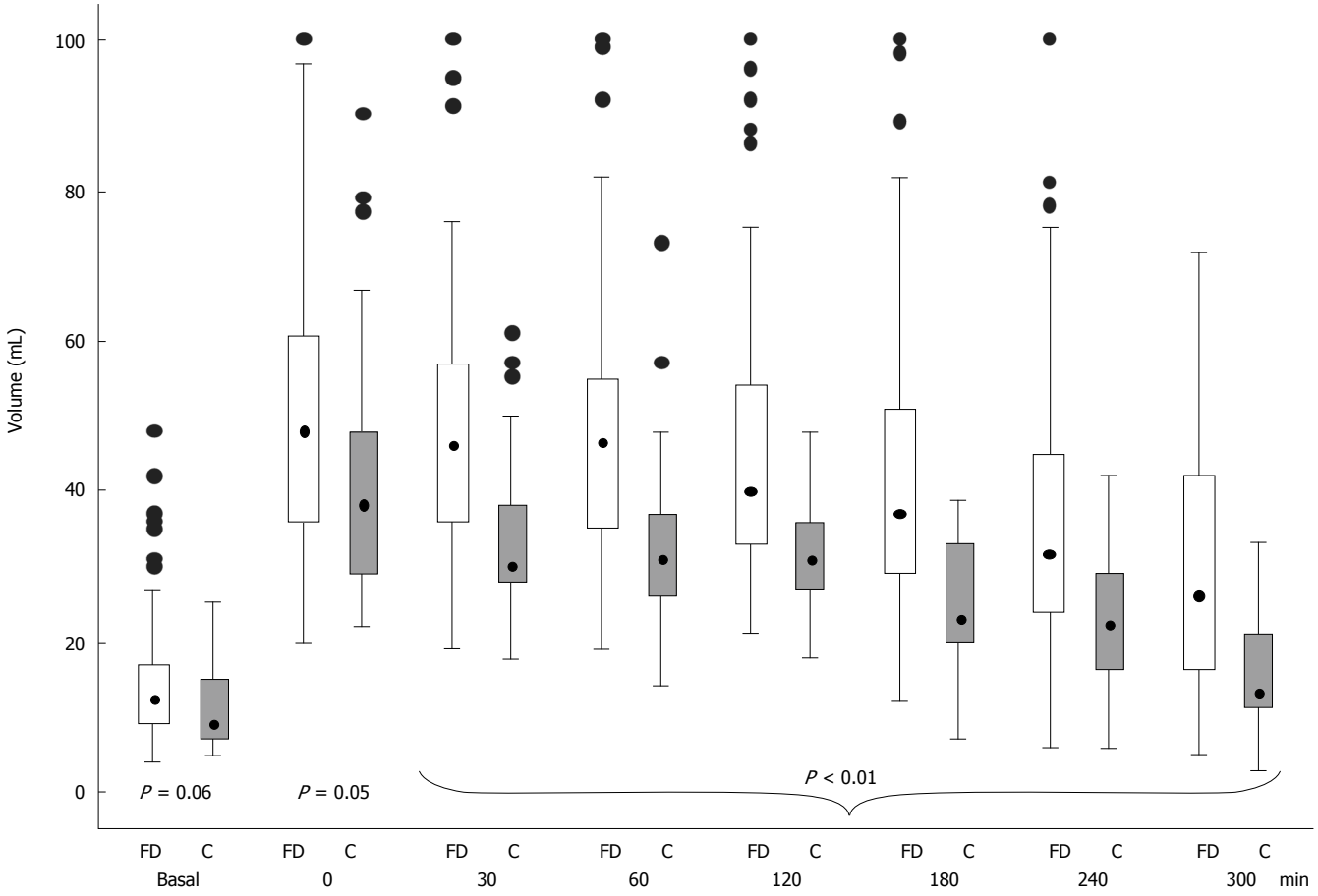

Figure 3 Box-and-whiskers plots of gastric antral volume before (fasting), immediately (0), at 30 and at 60 min intervals, after the end of the ingestion of a standard meal in controls (gray box) and in dyspeptic (white box). The boxes at each time unit extend from the 25th percentile ($x_{[25]}$) to the 75th percentile ($x_{[75]}$) [i.e., the interquartile range (IQ)]; the lines inside the boxes represent the median values. The lines emerging from the boxes (i.e., the "whiskers") extend to the upper and lower adjacent values. The upper adjacent value is defined as the largest data point $\leqslant x_{[75]} + 1.5 \times IQ$, and the lower adjacent value is defined as the smallest data point $\geqslant x_{[25]} - 1.5 \times IQ$. Observed values more extreme than the adjacent values, if any, are individually plotted (circles).

the antral volume transformations described in Figure 1 are reported in Table 3. The occurrence of postprandial drowsiness was related to (1) the current antral volume value, (2) the antral volume value at the previous time interval, (3) the antral volume delta variation between two consecutive measurements, (4) the mean postprandial weighted antral volume and (5) the maximal antral volume value reached after a meal. The occurrence of nausea and belching was related to the antral volume delta variation between two consecutive measurements. The occurrence of bloating was significantly related to a mean postprandial weighted antral volume, and marginally associated with (1) the current antral volume value, (2) the antral volume value at the previous time interval, and (3) the maximal antral

Table 4 AOR with 95% CI of having delayed gastric emptying on the basis of symptoms, fasting antral volume (FAV) and BMI

	AOR	95% CI	*P*
Drowsiness	3.59	1.12-11.54	0.03
Fasting antral volume[1]	1.09	1.02-1.17	0.007

AOR: Adjusted odds ratio; [1]AOR estimated per 5 mL increase (e.g. FAV of 20 mL *vs* 15 mL).

volume value reached after a meal.

Age was the only factor associated with the occurrence of some of the GI and extra-GI meal-related symptoms. Particularly, the occurrence of postprandial drowsiness was significantly associated with older age (AOR = 1.05 per 1 year increase, $P < 0.02$), while pain was marginally related to younger age (AOR = 0.96, $P < 0.04$). Epigastric burning was marginally related to older age (AOR = 1.11, $P < 0.03$).

The estimated AORs of having delayed gastric emptying are shown in Table 4. After a backward selection, only fasting antral volume and postprandial drowsiness were significantly associated with delayed gastric emptying, while no statistically significant relationship was found with age, gender and BMI. Fasting antral volume was significantly associated with delayed gastric emptying, increasing the OR of 93% for any additional volume increase of 5 mL.

DISCUSSION

An unexpected finding of this study was that more than 40% of symptomatic patients at the time of, and in the 3 wk preceding, the investigation did not refer any symptoms when challenged with a normal meal in controlled condition. The lack of any difference in the demography and symptom presentation between symptomatic and symptom-free patients during the investigation excludes a patient selection bias and indicates that the well known long-term variability[20] of dyspeptic symptoms may occur even over a short period of time.

Ultrasonography is a reliable method to estimate in normal physiological conditions gastric antral volume during fasting and after a meal[27-32]. The serial US measurements of the antral volume enable to assess directly the time curve of antral distension, and indirectly the gastric emptying time. Ricci *et al*[14] first reported a close association between the onset of the usual postprandial complaints and an antral volume increase in the majority of FD patients. Hausken *et al*[15] found an association between a wide antral area and bloating. Both studies, however, did not comparatively investigate the antral volume of the FD patients who remained symptom-free after meal ingestion.

Several other studies reporting non-univocal results have attempted to correlate the occurrence of symptoms in FD patients with proximal and distal stomach distension[10,11,17,36,37], gastric volumes[16,19,38-40] or gastric food retention[6,21,22]. These studies were performed at the end[16], 30 min[19] and 1 h[11] after meal ingestion, with either invasive techniques or not physiological ingestion of meals that caused dyspeptic symptoms also in healthy controls[16,17,19, 39,40].

This study assessed, in physiological conditions after a normal meal, the relationship between gastric emptying, antral volume variation and postprandial symptoms in symptomatic compared to asymptomatic dyspeptic patients and healthy subjects. The meal was a normal every day meal. Differing from previous findings at multivariate analysis, gender did not have any relationship with postprandial antral distention or with the modality of gastric emptying[22]. The different results of this study may be explained by the larger number of patients studied.

The antral volume reached its maximal value soon after the end of meal ingestion and subsequently decreased throughout the observation period in both controls and FD patients (Figure 3). However, independently from the concomitant presence of GI dyspeptic symptoms, FD patients showed an antral volume greater than healthy controls, confirming the presence of an altered intragastric meal distribution in FD patients[14-16,21,22]. It has been suggested that impaired accommodation of the proximal stomach to a meal underlies the increased distribution of gastric contents in the distal stomach[21,38].

The antral volume variations and gastric emptying were evaluated for a long postprandial period, i.e. 5 h, thus enabling to detect a subgroup of patients in whom the over distension of the antrum occurred later than, and independently from, an early postprandial altered motor function of the proximal stomach.

The most relevant finding of this study was that postprandial drowsiness was the only of the GI and extra-GI symptoms to be highly significantly associated with antral volume and its transformations.

In patients with postprandial drowsiness, antral volume reached the maximal value 2 h after the meal and did not show a progressive decrement throughout the observation period (Figure 4). The occurrence of postprandial drowsiness was significantly associated with the current and mean weighted antral volume, supporting the time-relationship with antral distension. However, the association with delta antral volume change suggests that its onset is associated with the degree of time-related antral distension rather than the antral volume *per se*. Finally, the finding of an association between drowsiness and the antral volume assessed at the preceding observation period suggests that the antral distension precedes the onset of drowsiness. It could be argued that the fasting and the immediate postprandial antral volumes included in the analysis might have inappropriately contributed to the subsequent antral volume transformation, therefore affecting the results of the present study, but their exclusion from the analyses did not change the results. Furthermore, it has been shown that subtracting the fasting from the final postprandial antral volume, the latter is still greater in dyspeptic patients than in controls, thereby indicating a genuine greater postprandial antral distension in patients compared to healthy controls[5].

At present, whether increased antral volumes may reflect hypotonia of the antral muscular wall or intraluminal distension secondary to gastric retention or to an overload caused by an impaired accommodation of the proximal stomach[21,38], or an increased duodenogastric reflux could

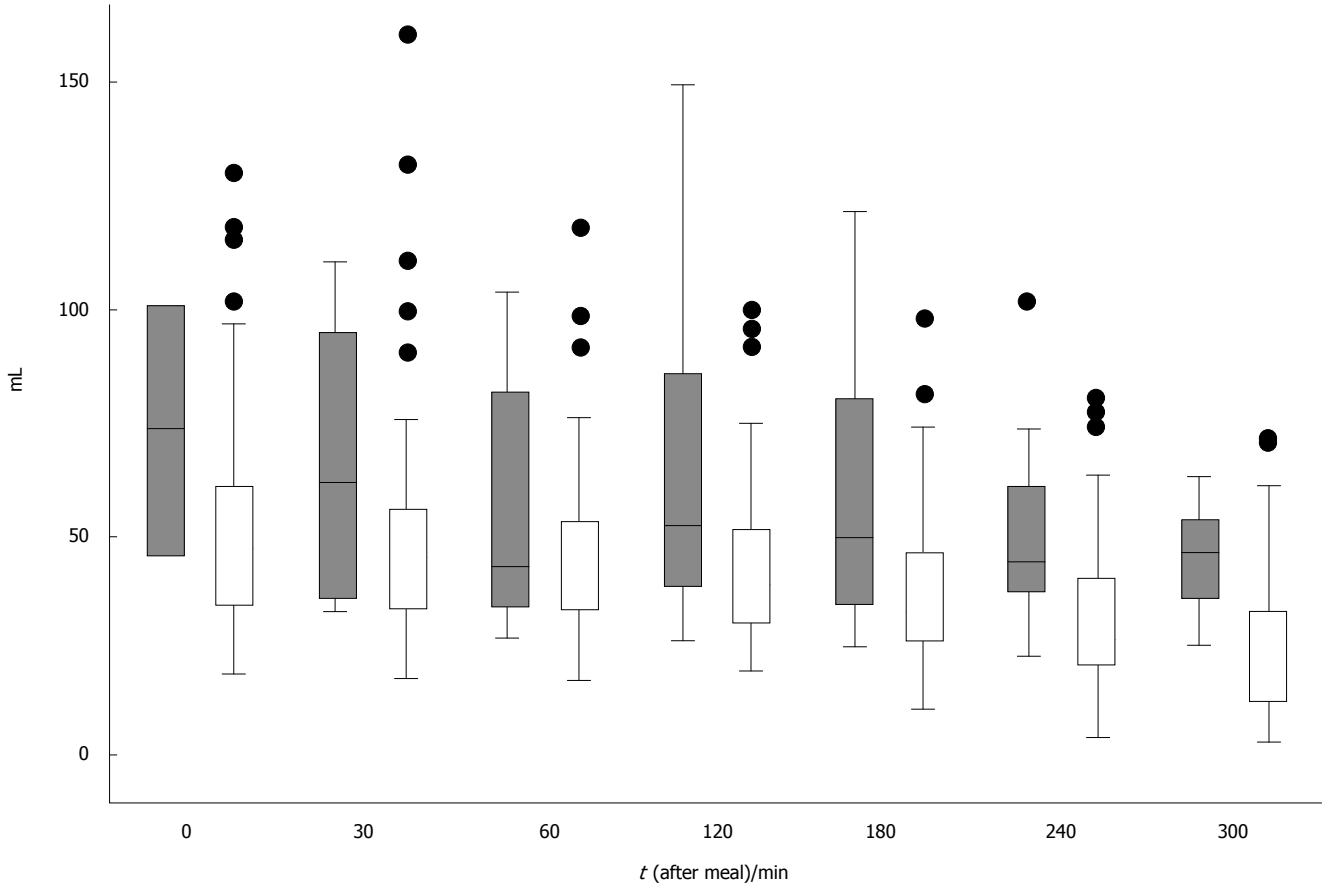

Figure 4 Box-and-whiskers plots of gastric antral volume immediately (0), at 30 and at 60 min intervals, after the end of the ingestion of a standard meal in FD patients with (gray box) and without (white box) postprandial drowsiness. The boxes at each time unit extend from the 25th percentile ($x_{[25]}$) to the 75th percentile ($x_{[75]}$) [i.e., the interquartile range (IQ)]; the lines inside the boxes represent the median values. The lines emerging from the boxes (i.e., the "whiskers") extend to the upper and lower adjacent values. The upper adjacent value is defined as the largest data point $\leq x_{[75]} + 1.5 \times IQ$, and the lower adjacent value is defined as the smallest data point $\geq x_{[25]} - 1.5 \times IQ$. Observed values more extreme than the adjacent values, if any, are individually plotted (circles).

not be addressed in the present study.

Several studies indicate that a variable degree of sleepiness after a meal is a common sensation[23,24,41-43] in healthy subjects and may also influence food intake[44]. Studies assessing daytime sleepiness with standard tests, in healthy subjects showed that (1) sleep onset latency was significantly shorter after a caloric meal than after water[24] and sham feeding[45], and (2) the occurrence of postprandial sleepiness was neither related to the fat composition of the test meal nor to the circadian variation in sleepiness[23]. Finally, in healthy subjects, it has been shown that in comparison with an equal volume of water and equicaloric liquid meal, a solid meal results in decreased sleep onset latencies[24]. These results, taken together with the observation that drowsiness may occur after intravenous administration[46] of cholecystokinin (CCK), support the hypothesis of a peripherally circulating hormone of GI origin that mediates postprandial sleepiness. It is conceivable that meal ingestion may release CCK and other neuroendocrine substances, such as 5-HT_3, that affect the state of consciousness directly or activating vagal nerve afferences or releasing other sleep-promoting substances, such as insulin[47,48]. Several animal studies indicate that CCK sleep-promoting and food intake-reducing effects are closely associated, presumably expressing different, yet related, manifestations of satiety[47].

Postprandial drowsiness reported in this study refers to a sensation regarded to be bothersome enough to interfere with the daily activities. However, the sensation of drowsiness may vary from slight to severe and may be related to sleep disturbances. Sleep disturbances were enquired specifically and patients reporting postprandial drowsiness did not report any sleep disturbances. In the present study, older age in FD patients was an independent factor adjusted for antral volume variables for the occurrence of drowsiness. Control subjects and dyspeptic patients were not balanced for age, however, in the age-adjusted model, the presence of drowsiness in FD patients was associated only with antral volume variations. In addition, it has been shown that in healthy subjects, postprandial drowsiness is related to a younger age and to a greater food intake compared to older subjects[44]. In the present study, healthy controls, although significantly younger than FD patients, did not report any symptom. It would therefore appear that, in contrast with healthy controls, drowsiness reported by older dyspeptic patients was neither related to sleepiness nor to physiological change of postprandial sleep latency. However, we neither evaluated with objective measures postprandial sleep latency or the severity of postprandial drowsiness nor we specifically evaluated physiological adaptive variation or an altered state of the autonomic nervous system that could

justify the occurrence of postprandial drowsiness in our patients[41].

Of the GI symptoms conventionally considered to be a manifestation of dyspepsia, only bloating showed a statistically significant association with the postprandial weighted antral volume and, to a lesser degree, with all the other antral volume variables, confirming previous observations[15,16,37]. Bloating has been frequently reported in healthy subjects in barostat studies, in caloric and even in water drink tests, without any relationship with altered gastric motor function[11,17,44]. In our study, nausea and belching were associated only with antral volume delta change, whereas all the other GI dyspeptic symptoms were not related to any of the assessed antral volume variables. Postprandial fullness is a common sensation even in healthy subjects and it has been reported to be associated with an increase of the antral area[49,50]. In the present study, none of the healthy subjects and about 50% of the study patient population referred fullness that was not associated with any of the antral volume variables evaluated. The frequent occurrence of bloating and postprandial fullness in healthy subjects together with the absence of a constant and univocal association with gastric functions limits the interpretation of the pathophysiological mechanisms of these symptoms in FD patients. Nausea is a non-specific GI symptom that could be elicited by the direct instillation of acid in the duodenum[51], i.e. a condition likely mimicking a physiological condition. Several studies highlighted the potential key role of the duodenum in the symptom generation in functional dyspepsia[51-53]. Hypersensitivity of the duodenum to acid infusion as well as duodenal distension have been reported to elicit upper GI symptoms[51-53]. However, the present study does not allow one to draw any conclusion about the role of the duodenum in the occurrence of symptoms and further investigations are needed.

Early satiety, which is one of the cardinal dyspeptic symptoms and was referred in the medical history by 45% of our patients population, did not occur after a meal in controlled condition in any of the investigated patients. It is conceivable that the sensation referred by the patients was more likely fullness or bloating that had induced the patients to terminate prematurely food ingestion to prevent further discomfort. It has been indeed shown that perception of fullness is a useful predictor of food intake[44,50]. If so, the definition of early satiety as an unpleasant sensation that forces one to stop eating, and the numerous studies in which this symptom has been related to specific function disorder of the stomach, should be reevaluated.

Nevertheless, a relevant limitation of this study is that except for pain and discomfort, severity of postprandial symptoms was not assessed, thus precluding any evaluation of its possible relationship with antral distension.

A significant proportion of FD patients complain of psychological symptoms and the presence of anxiety has been demonstrated in about 70% of these patients[22]. We excluded the patients with eating disorders and clinically evident psychological disorders like major depression from our study; however, we did not fully evaluate the psychological status of the patients.

Despite that it has been widely debated whether a delayed gastric emptying is a relevant factor in causing dyspeptic symptoms, to our knowledge, none of the previous studies evaluated concurrently and in physiological conditions the modality of the gastric emptying and the occurrence of symptoms in FD patients after an ordinary every day meal. In the present study, 39% of FD patients had delayed gastric emptying, but it did not have any relationship with the occurrence of dyspeptic symptoms during the test. A previous study reported a delayed gastric emptying in 41% of the patients and a rapid initial gastric emptying in 43% of them that were associated with higher symptoms score after a challenge meal[19]. Thus, it would appear that a delayed gastric emptying *per se* does not play any role in the origin of the usual dyspeptic symptoms.

The present study confirms our previous finding[5] that patients referring postprandial drowsiness have a greater probability to have delayed gastric emptying than controls and dyspeptic patients without post-prandial drowsiness. Differently from the usual dyspeptic symptoms, drowsiness is a late postprandial complaint occurring significantly later than GI symptoms and is related with antral distension and delayed gastric emptying. It would therefore appear that the two conditions are associated with the onset, and the persistence of postprandial drowsiness in this subgroup of FD patients. We cannot draw any definitive conclusion about the separate role, if any, played by delayed gastric emptying and antral distension, either alone or in combination, in the occurrence of postprandial drowsiness in FD patients.

In conclusion, our study assessed in controlled conditions the relationship between gastrointestinal and extra-gastrointestinal symptoms arising after a normal balanced meal, and antral distension and gastric emptying. Postprandial gastrointestinal symptoms do not have any constant or predictable relationship with antral distension and gastric emptying in functional dyspepsia patients. Of the extra-gastrointestinal symptoms, postprandial drowsiness is associated with antral distension. On the average, drowsiness occurs late after meal ingestion and after the onset of gastrointestinal dyspeptic symptoms. The onset of postprandial drowsiness is usually preceded by an increment of antral distension and the duration of the symptom appears to be related to the persistence of antral distension. Finally, a delayed gastric emptying is significantly associated with postprandial drowsiness and the degree of the fasting antral volume.

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CLINICAL RESEARCH

Milan criteria are useful predictors for favorable outcomes in hepatocellular carcinoma patients undergoing liver transplantation after transarterial chemoembolization

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Abstract

AIM: To evaluate whether the Milan criteria are useful in patients with hepatocellular carcinoma (HCC) who received transarterial chemoembolization (TACE) before liver transplantation (LT).

METHODS: Thirty-six HCC patients who fulfilled the Milan criteria after having received TACE and subsequently underwent LT were included (TACE + LT group) in the study. As controls, 21 patients who also met the Milan criteria and underwent LT without prior treatment were selected (LT group). Post-LT clinical outcomes, such as HCC recurrence, survival rate, and histologic features of explanted livers, were compared between the two groups.

RESULTS: Baseline characteristics were not different between the two groups. Pre-LT maximal tumor diameter in TACE + LT group was similar to that of LT group (2.0 ± 0.6 cm *vs* 2.3 ± 0.9 cm; $P = 0.10$). Post-LT histologic findings also revealed similar maximal tumor diameter in the two groups (2.4 ± 1.4 cm *vs* 2.3 ± 0.9 cm; $P = 0.70$). Explanted livers showed similar incidence of unfavorable pathologic features. The morality within 60 d after transplantation was not different between the two groups (8.3% *vs* 9.5%; $P = 0.99$). Post-LT 5-year survival rate (57% *vs* 74%; $P = 0.70$) and cumulative recurrence rate (8.3% *vs* 4.8%; $P = 0.90$) were not significantly different between the two groups.

CONCLUSION: The Milan criteria are still a useful selection criteria showing favorable outcomes in HCC patients receiving TACE before LT.

Key words: Milan criteria; Hepatocellular carcinoma; Transarterial chemoembolization; Liver transplantation

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INTRODUCTION

Hepatocellular carcinoma (HCC) is a major global health problem involving more than 500 000 new cases a year. Several treatment modalities, such as liver transplantation (LT), surgical resection, radiofrequency ablation (RFA), and percutaneous ethanol injection (PEI), are accepted for curative therapies for HCC. Theoretically, LT remains the only ideal treatment option because LT has been claimed to simultaneously cure the malignant disease and replace the premalignant cirrhotic liver. Early series of LT for HCC yielded poor outcomes[1-4]. In those series, 3- and 5-year survival after LT ranged 15%-67% and 15%-48%, respectively. These inferior results come from inclusion of patients with far advanced HCC. In spite of initial dismal experiences with LT for patients with HCC, patients with confined HCC (solitary lesion $\leqslant$ 5 cm or $\leqslant$ 3 lesions with diameter $\leqslant$ 3 cm, no major vessel invasion, and no extrahepatic involvement; Milan criteria) were reported to show an excellent long-term outcome with a 5-year survival rate of 70% and a recurrence rate below 15%[5]. With pathologic review, modestly expanded selection criteria (solitary lesion $\leqslant$ 6.5 cm or $\leqslant$ 3 lesions with the largest one $\leqslant$ 4.5 cm and total tumor diameter $\leqslant$ 8 cm; UCSF criteria) were suggested to offer an excellent outcomes with a 1- and 5-year survival rate of 90% and 75.2%, respectively[6]. In clinical practice, however, the Milan criteria based on pre-LT radiologic findings could be more useful and a widely accepted selection criteria than

the UCSF criteria based on post-LT pathologic findings.

Because of donor organ shortage or other limits including economic problem in HCC patients waiting for LT, various treatment modalities including resection, RFA, PEI, and transarterial chemoembolization (TACE) were tried to prevent the progression of HCC. Among these, TACE is the most commonly used procedure in patients with unresectable HCC in our country. Up to now, it is not known whether a favorable outcome after LT can also be achieved in HCC patients who have been treated by TACE and meet the Milan criteria, as in treatment-naïve HCC patients.

Hence, we conducted a study to assess the usefulness of the Milan criteria in HCC patients who had been treated with TACE prior to LT.

MATERIALS AND METHODS

Subjects

Between September 1996 and April 2004, a total of 105 patients with HCC underwent LT at our institute. Among them, 63 (59.4%) patients met the Milan criteria based on pre-LT imaging. Excluding 27 patients within Milan criteria treated by RFA or surgical resection before LT, 36 patients with one or more sessions of TACE only prior to LT were selected (TACE + LT group). Twenty-one HCC patients who had not been given any treatment before LT were selected as control (LT group) (Figure 1). The Milan criteria was defined as the presence of a tumor 5 cm or less in diameter in patients with single HCC or no more than 3 tumor nodules, each 3 cm or less in diameter, in patients with multiple tumors, and no extrahepatic metastasis, and no major hepatic vessel invasion[5].

TACE

One to eight sessions of TACE were performed *via* the transfemoral arterial approach under local anesthesia at intervals of 4-12 wk in the TACE + LT group. Selective celiac and superior mesenteric angiography was performed to define the hepatic artery anatomy and to evaluate the portal venous system. The feeding artery to the lesion was catheterized as selectively as possible using a highly flexible coaxial catheter, and the chemotherapeutic agent lipiodol mixture was injected under fluoroscopic guidance. The mixture contained 20-50 mg of doxorubicin, 3-20 mL of lipiodol (Lipiodol Ultrafluide; Guerbet, Aulnay-sous-Bis, France), and 3 mL of water-soluble contrast agent. Embolization was performed with gelatin pellets (Gelfoam; Upjohn, Kalamazoo, Michigan) thereafter in patients with liver function of Child-Pugh class A and tumor confined to single lobe of the liver. This Gelfoam embolization was also performed for patients with Child-Pugh B, if superselection of the feeding vessel was feasible. Abdominal computerized tomography (CT) scan was performed 4 wk after TACE to evaluate the anti-tumor effect of TACE, including lipiodol uptake by the tumor tissue. If viable tumor was still observed in CT, repeated TACE was performed. If lipiodol was compactly uptaken by all tumor nodules and any new lesion was not seen, follow-up CT scan was repeated every 3 mo.

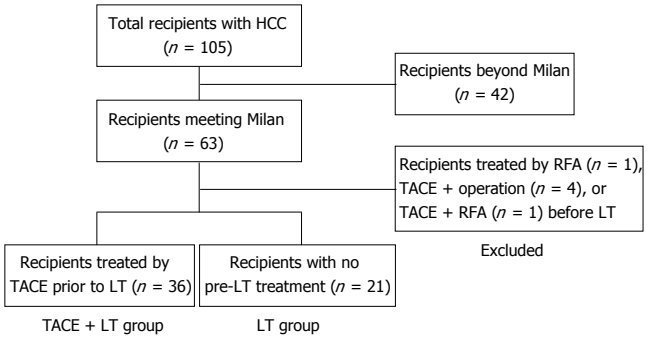

Figure 1 Selection of patients according to the treatments given prior to LT. Among a total of 105 HCC patients, 63 patients met the Milan criteria at the time of LT. After excluding 6 patients who received treatments other than TACE, there were 36 patients in TACE + LT group and 21 in LT group.

Pre-LT radiologic measurement of tumor size

As for tumor size in the LT group, any nodular lesion showing arterial enhancement and delayed washout in 3-phase helical CT was regarded as a viable HCC and its largest diameter was considered as a tumor size. In the TACE + LT group, a tumor nodule showing compact lipiodol uptake without arterial enhancement or delayed washout in CT was considered as non-viable tumor and was excluded from the measurement of tumor size or number. For a nodule showing arterial enhancement and delayed washout at the margin, tumor size was defined as the difference from diameter of the entire nodule to diameter of lipiodol-uptaken portion. Representative cases showing how to measure the HCC lesions treated by TACE are illustrated in Figure 2.

Histopathology

All total hepatectomy specimens were processed by a hepatopathologist using a routine protocol consisting of 1 cm or thinner sections throughout the entire liver. With standard histological staining, the specimens were examined to evaluate tumor characteristics, such as number of nodules, size, pathologic tumor grade (Edmonson grade), percentage of necrosis, and presence of tumor capsule invasion, satellite nodule, or microvascular invasion. By pathologic review of explanted liver, it was evaluated whether the enrolled patients also met the UCSF criteria.

Post-LT management and follow-up

Post-transplant immunosuppression consisted of corticosteroid plus either tacrolimus or cyclosporin. Corticosteroid was gradually tapered and was discontinued within 1 year. Tacrolimus and cyclosporin were continued after LT unless contraindicated. Acute rejection was treated with steroid pulse therapy. Antithymocyte globulin (ATG) or muromonab (OKT3) antibody infusions were reserved for the patients with acute rejection resistant to intravenous corticosteroids. The interval of the outpatient clinic visits after discharge from the hospital was adjusted according to the patient's condition.

Tumor recurrence was screened by measurement of alpha-fetoprotein and abdominal CT or ultrasonography

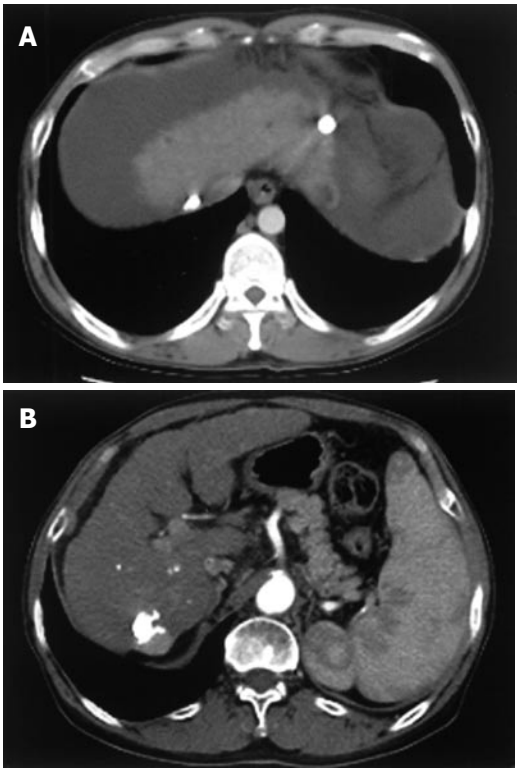

Figure 2 Representative cases for measurement of viable tumor size treated by TACE prior to LT. **A:** A tumor nodule with compact lipiodol uptake, no enhancement at arterial phase, and no washout at portal or delayed phase was considered as a non-viable tumor and was excluded from the measurement of tumor size or number; **B:** For a nodule showing arterial enhancement and delayed washout at the margin, tumor size was defined as the difference from diameter of the entire nodule to diameter of lipiodol-uptaken portion.

every 3 mo. Additional imaging modalities, such as chest CT and bone scan, were performed if HCC recurrence at lung or bone was suspected. No adjuvant chemotherapy was administered to any patients after LT.

Comparison of clinical outcomes

In this study, we retrospectively analyzed the medical records, radiologic and pathologic findings of the 36 patients in TACE + LT group and 21 in the LT group. Baseline clinical characteristics of the patients, tumor characteristics, clinical outcomes including recurrence of HCC, and post-LT survival rate were compared between the two groups.

Statistical analysis

Baseline characteristics of the patients were expressed as mean ± SD. Comparison between the two groups was done by using the independent t test for continuous variables and by chi-square test or Mann-Whitney U test for categorical variables. The overall survival rate and cumulative recurrence rate were calculated by the Kaplan-Meier method. The survival curves were compared by means of the log-rank test. All statistical tests were two-tailed and $P < 0.05$ was considered statistically significant. All statistical analyses were performed with SPSS 11.0 (SPSS Inc., Chicago, IL).

Table 1 Baseline clinical, demographic, and imaging-based tumor characteristics of the enrolled patients (mean ± SD)

Characteristics	TACE + LT group (n = 36)	LT group (n = 21)	P
Age (yr)	49 ± 8.2	52 ± 8.0	0.13
Sex (M/F)	31/5	18/3	0.99
Etiology of liver disease			0.12
HBV	34 (94.4%)	18 (85.7%)	
HCV	0 (0%)	2 (9.5%)	
HBV + HCV	1 (2.8%)	0 (0%)	
Alcoholic	0 (0%)	1 (4.8%)	
α-fetoprotein (μg/L)	193 ± 472	1012 ± 4110	0.24
TACE			
1	12 (33.3%)	-	
2	16 (44.4%)	-	
⩾ 3	8 (22.2%)	-	
MELD score	19 ± 9	22 ± 9	0.10
Type of graft			
Cadaveric graft	8 (22.2%)	2 (9.5%)	0.30
Living graft	28 (77.8%)	19 (90.5%)	
Number of nodules			
1/2/3	24/6/6	18/2/1	0.26
Diameter of the largest tumor (cm)			0.10
Mean ± SD	2.0 ± 0.6	2.3 ± 0.9	
Range	0.9-4.0	1.0-4.0	
Sum of the tumor diameters (cm)			0.38
Mean ± SD	2.5 ± 1.1	2.8 ± 1.3	
Range (cm)	0.9-6.1	1.0-5.9	

TACE: Transarterial chemoembolization; LT: Liver transplantation; HBV: Hepatitis B virus; HCV: Hepatitis C virus; MELD: Model for end stage liver disease.

RESULTS

Patient characteristics

A total of 57 patients (49 men and 8 women; median age 51 years, range 30-68 years) were included in this study. Fifty-three patients (93.0%) had HCC associated with hepatitis B virus (HBV) infection. Other causes consisted of hepatitis C virus (HCV) infection in 2 patients (3.5%), co-infection of HBV/HCV in one (1.8%), and alcohol-related liver disease in one (1.8%). The mean alpha-fetoprotein levels were 494.9 μg/L and model for end stage liver disease (MELD) score was 19.4. One to eight sessions of TACE were performed in TACE + LT group; 1 session in 12 (33.3%) patients, 2 sessions in 16 (44.4%) patients, and 3 or more sessions in 8 (22.2%) patients. Cadaveric grafts were used in 10 (17.5%) and living grafts in 47 (82.5%) cases. As for baseline clinical and demographic characteristics, there was no significant difference between TACE + LT group and LT group (Table 1).

Tumor characteristics

According to pre-LT radiologic evaluation, 42 (73.7%) patients had single tumor, and 8 (14.0%) and 7 (12.3%) patients had 2 and 3 tumors, respectively. The mean diameter of the largest nodule in all patients was 2.1 cm. In TACE + LT group, 24 (66.7%) patients had single tumor, 6 (16.7%) patients had 2 tumors, and 6 (16.7%)

Table 2 Histologic finding-based tumor profiles of patients who met the Milan criteria

Variable	TACE + LT group (n = 36)	LT group (n = 21)	P
Number of nodules			
1/2/3/≥4	18/8/3/7	13/3/3/2	0.79
Diameter of the largest tumor (cm)			
Mean ± SD	2.5 ± 1.4	2.3 ± 0.9	0.70
Range	0.6-7.5	0.5-4.2	
Sum of the tumor diameters (cm)			
Mean ± SD	3.8 ± 2.6	3.3 ± 1.3	0.36
Range	0.6-14.0	0.5-7.0	
Tumor differentiation[1]			0.59
Edmonson grade Ⅰ	5 (16.7%)	6 (28.6%)	
Ⅱ	22 (73.3%)	13 (61.9%)	
Ⅲ	3 (10%)	2 (9.5%)	
Presence of satellite nodule	5 (13.9%)	2 (9.5%)	0.99
Tumor capsule invasion	11 (30.6%)	3 (14.3%)	0.21
Microvascular invasion	11 (30.6%)	9 (42.9%)	0.40

[1]Assessment of tumor differentiation was possible in only 30 patients in TACE + LT group due to complete necrosis in 6 patients. TACE: Transarterial chemoembolization; LT: liver transplantation.

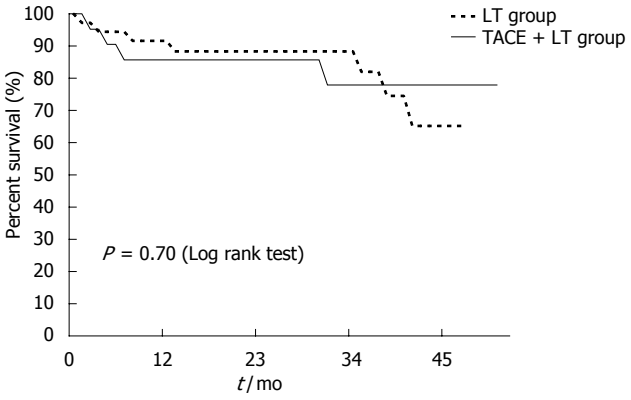

Figure 3 Comparison of overall survival rate between TACE + LT and LT groups. There was no obvious difference in 5-year survival rate after LT between the two groups (57% *vs* 74%; P = 0.70).

patients had 3 tumors. The distribution of tumor number was similar in the LT group; 1 in 18 (85.7%) patients, 2 in 2 (9.5%) patients, and 3 in 1 (9.5%) patient. No statistical difference was found in the mean diameter of the largest tumor between TACE + LT and LT groups (2.0 cm *vs* 2.3 cm; P = 0.10; Table 1).

According to histopathologic findings of explanted livers, a total of 114 nodules were found from all the patients; 77 in the TACE + LT group and 37 in the LT group. The number of tumor nodule was 1 in 31 (54.4%) patients, 2 in 8 (14.0%) patients, 3 in 3 (5.3%) patients, and 4 or more in 15 (26.3%) patients. The mean diameter of the largest tumor was 2.4 cm and the sum of the tumor diameters was 3.6 cm. There was no significant difference in distribution of tumor number between the two groups. The mean diameter of the largest tumor and the sum of the tumor diameters were also similar between both groups (2.4 cm *vs* 2.3 cm, P = 0.70; 3.8 cm *vs* 3.3 cm, P = 0.36, respectively, Table 2). Twenty-eight (77.8%) patients

Table 3 Detailed clinical data of the patients in whom HCC recurred after LT

Patient	Group	Time to recur (mo)	Site of recur	Treatment after recurrence	Survival (mo)
1	TACE + LT	9.6	Liver	None	10.0
2	TACE + LT	3.3	Liver, bone	TACE	7.1
3	LT	15.4	Lymph node	Excision, TACE	21.0
4	TACE + LT	0.9	Lung, liver	TACE	5.7

TACE: Transarterial chemoembolization; LT: Liver transplantation.

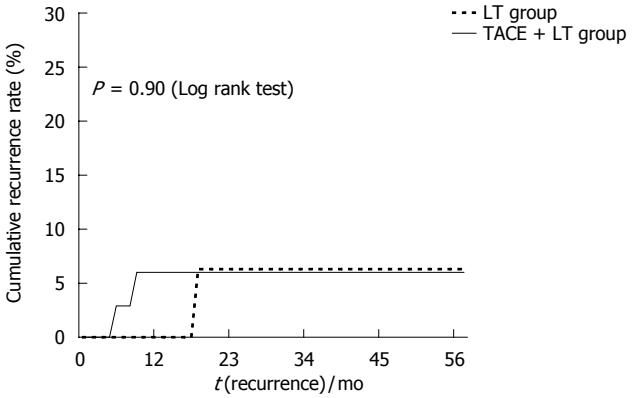

Figure 4 Comparison of cumulative HCC recurrence rate between TACE + LT and LT groups. Five-year recurrence rate was similar between the two groups (8.3% *vs* 4.8%; P = 0.90).

in the TACE + LT group and 19 (90.5%) in the LT group met the UCSF criteria.

As to the tumor differentiation, Edmonson grade 1 was found in 11 (21.6%), grade 2 in 35 (68.6%), and grade 3 in 5 (9.8%) patients. Of 57 patients, 7 (12.3%) and 14 (28.8%) patients had satellite nodules and tumor capsule invasion, respectively. In addition, 20 (35.1%) patients had microvascular invasion. The incidence of unfavorable pathologic features was similar in the two groups (Table 2). The explanted liver showed TACE-induced complete tumor necrosis without histologic evidence of viable carcinoma in 23 of 77 (29.9%) lesions.

Clinical outcomes after LT

During median follow-up of 24.3 (range: 0.1-99) mo, 14 of 57 (24.6%) patients died. Post-LT early mortality, defined as death within 60 d after transplantation, was not different between TACE + LT and LT groups (8.3% *vs* 9.5%; P = 0.99). The overall survival rates of the patients at 1-, 3-, and 5-year were 86%, 72%, and 67%, respectively. The 1-, 3-, 5-year survival rates between the TACE + LT group and the LT group were not significantly different (89% *vs* 81%, 68% *vs* 74%, and 57% *vs* 74%, respectively; P = 0.70) (Figure 3). The causes of death consisted of graft failure in 3 patients, graft *versus* host disease (GVHD) in 2 cases, hepatic vein problem with hepatic congestion in 1 case, HBV recurrence in 3 cases, HCC recurrence in 4 cases, and intracerebral hemorrhage in 1 case.

During the follow-up period, HCC recurrence was found in 4 (7.0%) patients (Table 3). Five-year cumulative HCC recurrence rate in the TACE + LT group was similar to that in the LT group (8.3% *vs* 4.8%; P = 0.90) (Figure 4).

DISCUSSION

HCC is one of the serious complications of chronic liver disease associated with high mortality. Although surgical resection is traditionally regarded as treatment of choice for HCC, it is a major problem that resection is feasible in only limited cases due to poor liver function and/or advanced stage of HCC at diagnosis. In addition, many patients suffer from recurrent HCC and aggravation of cirrhosis after operation. In this regard, LT gives patients with HCC an advantage over resection, because it addresses the multifocal potential of HCC in many patients that limits the success and applicability of resection and also treats the underlying liver disease[6].

The criteria developed by Mazzaferro and associates, known as the Milan criteria, have been widely applied around the world in the selection of patients with HCC for LT. However, the Milan criteria were originally made for patients with treatment-naïve HCC. In the clinical setting, a significant number of HCC patients are treated with TACE or RFA prior to LT because of a long waiting list for LT. It remains uncertain whether excellent outcomes can be obtained in HCC patients who previously underwent locoregional treatments and still meet the Milan criteria at the time of LT. In the present study, we retrospectively selected the patients who had received only TACE before they underwent LT since TACE is the most widely used procedure for HCC in our country. Patients undergoing other therapies, such as resection or RFA, were excluded to eliminate confounding effect of those treatments. For validation of usefulness of the Milan criteria in HCC patients treated with TACE before LT, their survival rates following LT were compared with those of HCC patients undergoing LT only.

The current study demonstrates that HCC patients who had undergone one or more sessions of prior TACE showed as good post-LT survival as treatment-naïve HCC patients, if they met the Milan criteria at the time of transplantation. One- and 5-year survival rates of patients in the TACE + LT group and LT group were comparable (89% *vs* 81% and 57% *vs* 74%, respectively; $P = 0.70$). Taniguchi *et al*[7] showed a long-term survival and marked TACE-induced tumor necrosis in patients with unresectable HCC. A recent report on randomized controlled trial showed that TACE with doxorubicin and gelatin sponge, compared with conservative management, provided survival benefits to patients with unresectable HCC[8]. However, as a bridge to transplantation for patients on waiting lists, TACE showed varying results[9,10]. Among these, an European study showed a 5-year survival of 93% in 48 patients receiving TACE, with no dropout over a mean waiting period of 6 mo[10]. Moreover, prospective studies have shown that the probability of preventing tumor progression is significantly higher in patients treated with TACE than those untreated controls[11,12]. In a recent series, pre-LT TACE in 54 predominantly early-stage cases yielded a reasonable 5-year post-LT survival of 74%[13]. However, the benefit of TACE could not be inferred given that waiting list dropout and post-LT recurrence rates were not markedly lower than historical controls. In contrast to previous studies regarding the role of TACE before LT, we elucidated the usefulness of the Milan criteria at the time of LT in patients receiving TACE prior to LT.

Up to now, there is no data on whether pre-LT TACE increases early mortality after transplantation compared to LT without prior therapy. In a previous study, some patients who underwent LT within 30 d of the last TACE developed unexplained severe pneumonia, leading to death very early after transplantation[9]. On the other hand, our results showed that mortality within 60 d after transplantation was not different between TACE + LT and the LT groups. Thus, the issue concerning pre-LT TACE and early mortality after operation still remains controversial.

In our study, 1-year survival rates of LT group were lower than those of TACE + LT group, albeit not statistically significant (89% *vs* 81%). Although the reason for this observation is not clearly understood, it might be due to the poorer liver function of patients in LT group as compared with the TACE + LT group. Urgent transplantations might have been undertaken in patients of the LT group whose liver functions were too poor to perform locoregional therapy. The tendency of higher MELD score (22 *vs* 19; $P = 0.10$) and more frequent living donor transplantation in the LT group compared to the TACE + LT group (90.5% *vs* 77.8%; $P = 0.30$) support the more aggravated liver function and urgent condition of the LT group.

Our data demonstrated that 5-year cumulative recurrence rates of patients in the TACE + LT group were similar to those in the LT group (8.3% *vs* 4.8%; $P = 0.90$). HCC recurred in 3 of 36 patients in the TACE + LT group with a recurrence site of liver in one and distant organs in two patients. In the LT group, the recurrence occurred in one patient at the perihepatic lymph node. Increased incidence of hepatic or extrahepatic recurrence after TACE followed by resection has been an important concern on the grounds that partial necrosis of the tumor favors the shedding of neoplastic cells in a few previous studies[14-17]. However, such a concern was not substantiated in our patients.

There was no significant difference between the TACE + LT group and the LT group in terms of baseline clinical and demographic characteristics, radiologic finding-based or histologic finding-based tumor profiles, and histologic parameters indicating unfavorable prognosis. At this point, we have to comment on the radiologic measurement of HCC lesions previously treated with TACE. In the TACE + LT group, albeit statistically not significant, the radiologically measured diameter of the largest tumor was shorter than the histologically measured one (2.0 ± 0.6 cm *vs* 2.5 ± 1.4 cm; $P = 0.06$), and 4 of 36 patients in the TACE + LT group were found to have 4 or more nodules on explanted specimens. In the LT group, the radiologically measured diameter of the largest tumor was similar with the histologically measured one (2.3 ± 0.9 cm for both), and 2 of 21 patients in the LT group had 4 or more nodules on explanted livers. These results imply that pre-LT staging of HCC by the current imaging modalities may be underestimated in terms of number in both groups and in terms of size in the TACE + LT group.

Although the extents of HCCs were estimated by

expert radiologists using a pre-defined method, it was difficult to precisely measure the maximal diameter of lesions in a portion of the TACE + LT group, especially in cases with incomplete or scattered lipiodol uptake in nodules. In a previous study measuring the tumor volume (TV) to improve the selection criteria based on number and diameter of HCC, TV > 28 cm^3 was reported to be a predictive factor for HCC recurrence after LT[18]. Other investigators measuring the TV with a region-of-interest CT technique also evaluated the prognostic value of volumetric CT in patients treated with repeated TACE[19]. These alternative methods might better represent the tumor size, but are much more complex and time-consuming.

In conclusion, our data demonstrated that the Milan criteria would be useful selection criteria in HCC patients who underwent the TACE procedure before LT, showing favorable prognosis if they fulfill the criteria at the time of transplantation.

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CLINICAL RESEARCH

mRNA expression, functional profiling and multivariate classification of colon biopsy specimen by cDNA overall glass microarray

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Abstract

AIM: To understand the local pathophysiological alterations and gene ontology-based functional classification of colonic biopsies into inflammatory and neoplastic diseases.

METHODS: Total RNA was extracted from frozen biopsies and amplified by T7-method. Expression profile was evaluated by Atlas Glass 1K microarrays. After microarray quality control, applicable data were available from 10 adenomas, 6 colorectal adenocarcinomas (CRCs), and 6 inflammatory bowel diseases (IBDs). Multivariate statistical and cell functional analyses were performed. Real-time RT-PCR and immunohistochemistry were used for validation.

RESULTS: Discriminant analysis of selected genes, could correctly reclassify all 22 samples using 4 parameters (heat shock transcription factor-1, bystin-like, calgranulin-A, TRAIL receptor 3). IBD samples were characterized by overregulated chemokine (C-X-C motif) ligand 13, replication protein A1, E74-like factor 2 and downregulated TNF receptor-associated factor 6, BCL2-interacting killer genes. In adenomas upregulation of TNF receptor-associated factor 6, replication protein A1, E74-like factor 2 and underexpression of BCL2-associated X protein, calgranulin-A genes were found. CRC cases had significantly increased epidermal growth factor receptor, topoisomerase-1, v-jun, TNF receptor-associated factor 6 and TRAIL receptor 3, and decreased RAD51 and RAD52 DNA repair gene, protein phosphatase-2A and BCL2-interacting killer mRNA levels. Epidermal growth factor receptor RT-PCR and immunohistochemistry, topoisomerase-1 RT-PCR confirmed the chip results.

CONCLUSION: Different histological alterations can be reclassified by functional, multivariate analysis using cDNA microarrays. Further studies with expanded sample number are needed for subclassification of pathological alterations.

Key words: Adenoma; Biopsy samples; Colorectal cancer; Gene expression; Inflammatory bowel diseases; Microarray technology

Galamb O, Sipos F, Dinya E, Spisak S, Tulassay Z, Molnar B. mRNA expression, functional profiling and multivariate classification of colon biopsy specimen by cDNA overall glass microarray. *World J Gastroenterol* 2006; 12(43): 6998-7006

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INTRODUCTION

mRNA expression array analysis is usually performed on high volume surgery or blood samples. However, evaluation of routine biopsy specimens could yield information, as to how the local pathological processes differ from healthy counterparts. In the gastrointestinal tract, biopsy samples are routinely taken. The mRNA expression study of these samples could allow further insight into the development of inflammatory, preneoplastic and neoplastic diseases.

These specimens could not be applied previously for expression array studies, because array technology even today needs significantly more RNA than can be isolated from the tiny biopsy specimen. However, new techniques and commercial kits have recently become available for the reliable mRNA amplification without an effect on the original gene expression pattern[1].

Previous microarray analyses reported in the literature were performed predominantly from surgically resected colon adenocarcinoma samples[2], with gene expression analysis of colonic biopsies done in only two cases[3,4].

In a few studies, local pathological alterations were examined using cDNA microarrays in cells and tissue structures laser-microdissected from surgical material[4,5]. The mRNA expression patterns of tumorous and normal tis-

sues were usually compared by cDNA microarrays[5,7-11] or oligonucleotide microarrays[12,13] containing some hundreds to several thousand fixed target sequences.

Microarray gene expression profiling of adenomas as a precancerous stage of colon adenocarcinoma is less represented in the scientific literature. Oligonucleotide or cDNA microarray-based molecular diagnostics of malignancy in colon adenoma and colorectal cancer samples was described using 10[14], 9[6] and 4[15] adenoma samples compared to adenocarcinoma and normal colonic tissues.

Inflammatory bowel diseases (IBDs) were rarely analyzed by microarrays. Lawrance *et al*[16] published an oligonucleotide microarray comparison between surgically resected human ulcerative colitis (UC), Crohn's disease (CD) and normal colonic tissues. Recently, Langmann *et al*[3] used mucosal biopsy specimens for global gene expression profiling in patients with UC and CD.

cDNA-based mid-sized (1K) commercial arrays have recently appeared, which do not need any special hybridization, washing or scanner apparatus. These open-platform arrays, if of good quality and reproducibility, can contribute to a widespread use of the array technology. Mid-sized so called "overall" microarrays with fixed target cDNAs from the most important cell processes (adhesion, apoptosis, cell cycle, DNA replication and repair, extracellular matrix remodeling, cytoskeleton, immune regulation, metabolism, stress response, oncogenesis and tumor suppression, growth factor-related cell proliferation, neuroendocrine regulation, signal transduction, transcription and transport) give us opportunity for analysis of different cell functions that may be associated with diseases.

Bioinformatical analysis of these types of arrays can then be used to detect the expression pattern differences between limited numbers of diagnostic groups. Multivariate statistical analysis methods can be applied for the development of automated classification methods like image analysis based cervical cancer screening[17,18].

In the present study we aimed to prove that normal, inflammatory, premalignant and malignant colon biopsies that can be used for mRNA expression analysis and the expression differences can be utilized in a multivariate classification system. As colorectal adenocarcinoma (CRC) frequently arises in the setting of various high-risk conditions such as adenomatous polyps and IBD, we looked for gene expression pattern-based connections between these types of diseases.

MATERIALS AND METHODS

Patients and samples

Routine biopsy specimens were collected from the pathological and normal part of the colon, placed in RNALater Stabilization Reagent. Total RNA was extracted from frozen biopsy specimens from 11 patients with adenomatous polyps, 12 with CRC and 11 with IBDs (5 UC, 6 CD). After quality control of the microarrays, applicable data were available from 10 patients with adenomatous polyps, 6 with CRC and 6 with IBD (3 UC, 3 CD).

Four male and two female patients of 53-86 years (median, 72.17 years) with CRC were involved in the study. Five patients had left side (2 rectal, 33%; 3 sigmoid , 50%) and 1 had right side (coecal, 17%) involvement. Two patients had localized disease without nodal or distant organic involvement (Dukes B stage, 33%), 1 patient had nodal involvement (17%, Dukes C stage) and 3 had liver metastases (50%, Dukes D stage).

Nine male and one female patient of 17-77 years (median, 61.2 years) with colorectal adenoma were involved in the study. Five of them had left side (1 rectal, 10%; 3 sigmoid, 30%; 1 descendent colonic, 10%), and 5 had right side (2 ascendant colonic, 20%; 1 coecal, 10%; 2 total colonic, 20%) involvement. Six patients had tubular (60%), 2 had tubulovillous (20%) and 2 had villous adenoma (20%). Only one of the villous adenoma patients' biopsy sample contained severe dysplastic alteration.

One male and five female IBD patients of 23-72 years. (42 years. median) were involved in the study. Three of them [50%; 1 UC (33%) and 2 CD (66%)] had total and three [50%; 2 UC (66%) and 1 CD (33%)] had left side colonic disease. The grade of inflammation was severe in 3 cases (50%) and moderate in 3 cases (50%).

Methods

Total RNA isolation using Qiagen RNeasy Mini Kit: Frozen biopsy samples were lysed and homogenized in a mixture of 300 μL GITC-containing lysis buffer and 3 μL β-mercaptoethanol by Polytron homogenizator for 30-40 s. The lysed samples were digested in proteinase K solution at 55℃ for 10 min. After silica membrane cleaning, according to the manufacturer's description and DNase I treatment (in order to absolutely remove genomic DNA), the total RNA was eluted in 50 μL RNase-free water. Quantity and quality of the isolated RNA was tested by measuring the UV absorbance, by using real-time RT-PCR (Light Cycler G6PDH Housekeeping Gene Set, Roche) and by agarose gel electrophoresis. The high quality, intact total RNA samples, which showed regular 18S and 28S ribosomal RNA bend pattern during the agarose gel analysis, and showed positive real-time RT-PCR reaction were used for microarray analysis.

T7 RNA amplification and labeling: Because the total RNA content of the biopsy samples was lower than 10 μg, which was not enough for one hybridization reaction-the mRNA fraction was amplified by the T7-method (MessageAmp Ⅰ aRNA Kit, Ambion Inc., US), according to the manufacturer's instructions, fluorescently labeled probes were synthesized using amino allyl UTPs and Cy-3 and Cy-5 monoreactive dyes (Amersham Biosciences Ltd., England).

Atlas Glass 1.0K microarray analysis: The mRNA expression profile was evaluated by Atlas Glass microarrays (BD Clontech Inc. US, 1081 genes). Two hundred μL fluorescently labeled probes (mixture of the appropriate Cy3- and Cy5-labelled cDNA) were mixed with the prewarmed hybridization solution and hybridized to the microarray for 16 h at 50℃. Washing steps were done at room temperature in washing solutions (containing 0.75 mmol/L DTT) according to the manufacturer's instructions. The slides were dried by blowing with carbone-dioxyde and were scanned by Axon GenePix4000B reader on 532 nm (Cy3) and 635 nm (Cy5) wavelengths.

Data analysis: Scanned arrays were evaluated by the GenePix Pro 4.1 software. Automated spot detection using local background determination was done and feature extraction (ratio of medians, ratio of means, Cy3/Cy5) was performed. Microarrrays with nonhomogeneous background and/or incomplete housekeeping gene spot set were removed from further analysis. Ratios of medians of the detected features were applied in the normalization. Means of the ratio of medians were normalised to be 1. Results were exported into Acquity 3.1 software (Axon Inc.) and datasets were established. Dataset selection into classification categories was performed. Mean, median, SD of each parameter was calculated. Underexpression was defined as ratio of means ± SD < 0.5, overexpression as ratio of means ± SD > 2.0. One-way (group) ANOVA and multivariate exploratory techniques (discriminant analysis, factor analysis and hierarchical cluster analysis) were performed by SAS 6.12 version statistical software. Hierarchical cluster analysis was done using Ward's method (Euclidean distances). The colonic cases were clustered according to their expression pattern based on the filtered ANOVA results. Gene annotation and functional classification were done using Atlas Gene List Version 4.0. Functional analysis and visualization of biological association network were done using Pathway assist 2.53 software.

Validation: Real-time RT-PCR was used for validation of expression microarray results (Roche LightCycler). One-step RT-PCR was carried out using the LightCycler h-β2M Housekeeping Gene Set and RNA Master Hybridization Probes kit. For the relative quantification, commercially available β-2-microglobulin (142 bp length fragment) was used as a reference, and newly designed and synthesized epidermal growth factor receptor (EGFR) (227 bp length fragment) or DNA topoisomerase I (Top1) (184 bp length fragment) were used as a target gene. The following primer and hybridization probe sequences were used: EGFR9 S primer: 5'-atcctgccggtggcatt-3', EGFR12 A primer: 5' -gttcaggctgacgactgca-3', EGFR-FL probe: 5'-caggacggac ctccatgcctttga-3', EGFR-LC probe: 5'-LC Red 640-cctag aaatcatacgcggcaggacc-3' in case of EGFR, and Top1-fw primer: 5'-acatcatgcttaaccctagttcac-3', Top1-as primer: 5' -cagagcaagcttgtcgatg-3', TOP1-FL probe: 5'-cggatcttgtcc acacatttttttcagc-3', TOP1-LC probe: 5'-LC Red 640-ccgag cagtctcgtatttctgccag-3' in case of DNA-topoisomerase-I. Evaluation of relative ratios (diseased/normal/same patient) was prepared using RelQuant software.

EGFR immunohistochemistry: Formalin fixed paraffin embedded 4 μm thick colonic biopsy tissue sections were dewaxed and rehydrated. Antigen unmasking was carried out by nuclease free Proteinase K digestion for 20 min at room temperature. After washing twice in PBS, endogenous peroxidase activity was blocked by incubation in 3% hydrogen peroxide for 30 min at room temperature. After washing 3 times in PBS for 3 min, a specific blocking was done with 1% BSA-PBS solution for 10 min at room temperature. Then the slides were incubated with diluted EGFR culture supernatant antibody (1 μL EGFR antibody and 40 μL PBS) (Clone: H-11, DAKO) at 37℃ for 60 min in a humidified chamber. After washing 3 times in PBS, signal conversion was carried out with the LSAB2 system (DAKO) according to the manufacturer's instructions. Haematoxylin co-staining was done.

Ethical consideration: All routine colonic biopsy specimens from the patients were taken after informed consent and ethical permission was obtained for participation in the study.

RESULTS

Identification of commonly over-and underexpressed genes in colonic diseases

Genes that were up- and downregulated in at least 2/3 of cases per sample group in colorectal cancer (Supplemental Table 1), in adenoma (Supplemental Table 2) and in IBD (Supplemental Table 3) were considered as a commonly over- and underexpressed genes. CRC cases are characterized by upregulated genes in the DNA replication (such as replication protein A1, DNA topoisomerase Ⅱα, DNA topoisomerase Ⅰ), cell cycle (including cyclin A1, cyclin-dependent kinase 10, protein NIMA-interacting 1), extracellular matrix remodeling (like keratin 5, perlecan, enactin), transcription regulation (such as IRF5, 6, and E74-like factor 2) , oncogenesis (including v-jun, BRCA2) and growth factor related cell proliferation (EGFR, VEGFB, hepatocyte growth factor, transforming growth factor β2) cell function groups; and downregulated genes in the DNA repair (RAD51 and 52 homolog), tumor suppression and apoptosis (like BCL2-interacting killer) cell function groups. Adenoma cases in comparison showed altered gene expression data in apoptosis (such as TNF receptor-associated factor 6, BAX), growth factors, receptors and their signal transduction (like calgranulin A, KIT ligand, Ran GTPase activating protein 1), oncogenesis and tumor suppression (including growth factor receptor-bound protein 10, p53-induced protein and betaglycan) functional groups. IBD cases are featured by the gene expression changes of immune regulation (including GM-CSF2, CXCL13, MMP-3, MMP-12 and interleukin 1 receptor antagonist), transport [like transferring and solute carrier family 25 (mitochondrial carrier; adenine nucleotide translocator), member 5], and growth factor-related cell proliferation (such as B cell growth factor 1, GM-CSF).

The most differentially expressed genes between the 3 sample groups in colon-filtered results of ANOVA

We analyzed the expression differences between all 1081 genes using ANOVA. The results of ANOVA were filtered according to the number of cases. The genes without enough data from all colonic samples were removed from the analysis. 19 genes were found to be significantly differently expressed ($P < 0.05$) between the colonic sample groups using filtered ANOVA method (Table 1).

Factor analysis and discriminant analysis

Factor analysis was prepared on the basis of the results of variance analysis. Factor analysis resulted in two different factor groups. Factor 1 had the most considerable explorative variance value (6.331196), but the factor 2 also showed significant explorative variance values (4.710563). The factor analysis gives information about the functional gene groups which can differentiate the observed diseases

Table 1 The most differentially expressed genes between the 3 sample groups in the colon

Name	GenBank ID	Differences between sample groups	*P*	Number of valid cases/ sample groups	Ratio of means[1] in adenoma	Ratio of means[1] in CRC	Ratio of means[1] in IBD	Cell function
Adhesion								
Bystin-like	L36720	1-3 2-3	0.0005 0.0008	5, 2, 2	0.612 ± 0.62676	1.47 ± 0.789131	7.3375 ± 1.134906	Other cell adhesion proteins
Apoptosis								
Tumor necrosis factor receptor superfamily, member 10c, decoy without an intracellular domain	AF016267	1-2 2-3	0.0044 0.0046	4, 4, 5	0.49075 ± 0.315247	2.7695 ± 1.308503	0.5032 ± 0.270649	Death domain receptors
Extracellular matrix, cytoskeleton								
Matrix metalloproteinase 13 (collagenase 3)	X75308	2-3	0.0395	10, 6, 6	0.8075 ± 0.406413	0.557 ± 0.324015	1.222333 ± 0.558757	Metallo proteinases
Metabolism, blood coagulation								
Ubiquitin C	M26880	1-3 2-3	0.0325 0.0227	10, 5, 5	0.9329 ± 0.687387	0.8456 ± 0.51739	2.3002 ± 1.110219	Protein turnover
Plasminogen activator, urokinase	M15476	1-3	0.0089	6, 5, 4	0.482167 ± 0.248489	1.0554 ± 0.524046	1.513 ± 0.417319	Serine proteases
Oncogenes and tumor suppression								
AXL receptor Tyrosine kinase	M76125	1-2	0.0209	7, 5, 4	0.258571 ± 0.237985	2.0162 ± 1.429626	1.3225 ± 0.780286	Oncogenes and umor suppressors
Ras homolog gene family, member H	Z35227	1-2	0.0076	7, 5, 3	0.806429 ± 0.732179	2.736 ± 1.083434	1.115 ± 0.249638	Oncogenes and tumor suppressors
RAB5A, member RAS oncogene family	M28215	1-3	0.0151	8, 5, 5	0.5745 ± 0.438766	0.9958 ± 1.224974	2.263 ± 0.863959	General trafficking
Receptors and ligands								
Small inducible cytokine A5 (RANTES)	M21121	1-2	0.0087	8, 4, 6	0.706125 ± 0.402215	1.94025 ± 0.799844	1.0615 ± 0.371365	Growth factors, cytokines, and chemokines
Pleiotrophin (heparin binding growth factor 8)	M57399	1-2	0.0373	5, 5, 2	0.3474 ± 0.242632	2.7236 ± 1.872401	0.477 ± 0.166877	Growth factors, cytokines, and chemokines
Patched (Drosophila) homolog	U43148	1-3 2-3	0.0043 0.0058	9, 5, 5	0.653667 ± 0.463297	0.7752 ± 0.448416	3.8946 ± 2.578342	Growth factor and chemokine receptors
Stem cell growth factor; lymphocyte secreted C-type lectin	D86586	1-2	0.01755	5, 5, 4	0.3264 ± 0.168316	1.6386 ± 0.957384	0.5585 ± 0.420226	Ggrowth factors, cytokines, and chemokines
Interleukin 7 receptor	M29696	1-2 2-3	0.0096 0.0066	8, 4, 4	0.40775 ± 0.12511	1.774 ± 1.113227	0.32975 ± 0.133809	Interleukin and interferon receptors
Signal transduction								
Myotonic dystrophy protein kinase like protein	Y12337	1-2 1-3	0.0253 0.0131	10, 6, 6	0.7611 ± 0.296331	1.375167 ± 0.301345	1.4415 ± 0.521753	Intracellular kinase network members
Transcription								
Heat shock transcription factor 1	M64673	1-2	0.0136	9, 6, 3	0.732444 ± 0.421368	1.7565 ± 0.747317	0.385333 ± 0.320644	Transcription activators and repressors
Immediate early protein	M62831	2-3	0.0209	8, 5, 5	0.552875 ± 0.271426	0.4708 ± 0.276956	0.989 ± 0.255144	Basic transcription factors
High-mobility group (nonhistone chromosomal) protein isoforms I and Y	M23619	1-3 2-3	0.0003 0.0011	9, 6, 5	0.734556 ± 0.324623	0.897667 ± 0.371581	1.7982 ± 0.238334	Chromatin proteins

[1]Cy3/Cy5 (disease/normal).

according to their different expression levels (Table 2).

According to the expression changes of the following genes the three colonic disease groups can be significanty distinguished: HSF1 (P = 0.012537), bystin-like (P = 0.001027), calgranulin A (P = 0.043831), and TNFR superfamily member 10c (P = 0.037888) (Figure 1).

Cluster analysis

Four different clusters can be identified: two IBD-related clusters containing one CRC case, a carcinoma group containing one adenoma and one ulcerative colitis case, and an adenoma cluster with nine adenoma cases (Figure 2). Tree diagram of 22 colonic cases showed considerable accordance with the conventional histopathological diagnoses. Excluding one case (AD1), all adenoma cases belong to a significantly distinct cluster. The cluster of severely inflammed IBD cases contained one colorectal adenocarcinoma case, as it showed a similar gene expression pattern. This

Table 2 Results of the factor analysis

STAT. factor analysis	Factor loadings (Varimax raw) Extraction: Principal components (Marked loadings > 0.700000)		Cell function
	Factor 1	Factor 2	
Stem cell growth factor	0.896313	-0.053631	Growth factors, cytokines,and chemokines
Pleiotrophin (heparin binding growth factor 8)	0.882657	0.006966	Growth factors, cytokines, and chemokines
Small inducible cytokine A5 (RANTES)	0.802949	0.169587	Growth factors, cytokines, and chemokines
Interleukin 7 receptor	0.835349	-0.151447	Interleukin and interferon receptors
Tumor necrosis factor receptor superfamily, member 10c	0.742897	-0.145755	Death domain receptors
Signal transducer and activator of transcription 2113 kDa	0.820315	0.066954	Transcription activators and repressors
Heat shock transcription factor 1	0.745691	0.032620	Transcription activators and repressors
Patched (Drosophila) homolog	-0.113082	0.768421	Growth factor and chemokine receptors
RAB5A, member RAS oncogene family	-0.044311	0.834146	General trafficking
Ubiquitin C	-0.045668	0.719247	Protein turnover
High-mobility group protein isoforms I and Y	-0.029882	0.943945	Chromatin proteins
Explorative variance	6.331196	4.710563	

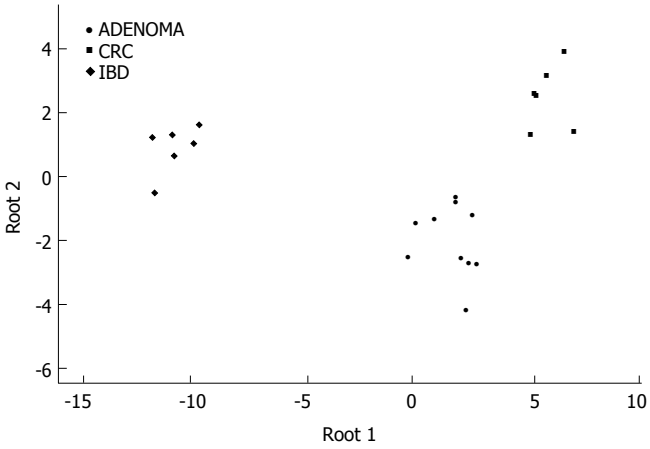

Figure 1 Discriminant analysis of colonic biopsy specimens. Note the clear separation of the single classification groups based on the discriminatory genes detailed in the results section.

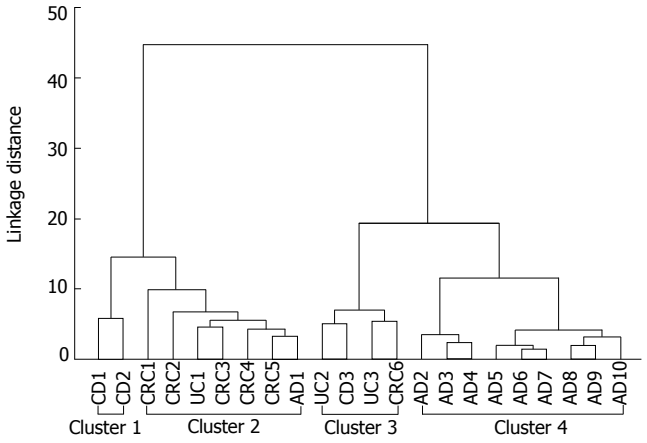

Figure 2 Tree diagram for 22 colonic diseases (Ward's method euclideandistances). Four different clusters can be identified: 1 and 3 are IBD-related clusters containing one CRC case; 2 is a carcinoma group containing one adenoma and one ulcerative colitis cases; 4 is an adenoma cluster with nine adenoma cases. AD: Adenoma; CD: Crohn's disease; CRC: Colorectal cancer; UC: Ulcerative colitis.

observation could reflect that colorectal cancer can develop from chronic inflammation and some types of adenomas.

Validation

All samples with remaining total RNA (9 CRCs, 2 adenomas and 3 CD samples), were tested using one-step EGFR and TOP1 real-time RT-PCR. Using β-2-microglobulin as a standard control, EGFR- and TOP1 mRNA levels were measured, and ratios of these concentrations to the standard were determined. Ratios for each disease group along with normal samples were compared, and the resulting relative ratios determined (diseased/normal in the same patient). The CRC samples showed higher EGFR mRNA level than the normal paired sample (relative ratio: 3.008, SD: 4.591), while there was no differential expression found in the adenoma samples (relative ratio: 0.772, SD: 0.060). Lower EGFR expression was measured in CD samples (relative ratio: 0.451, SD: 0.173) compared to the normal adjacent mucosa. Increased, 2.802-fold TOP1 mRNA level was evaluated in the CRC samples (SD: 3.884), but TOP1 level of the other sample groups was within the normal range (realtive ratio-adenoma: 0.774, SD: 0.0368; realtive ratio-CD: 0.942, SD: 0.337). The expression differences of EGFR and TOP1, found by microarray analysis, could be confirmed in most, but not in all cases. First, because of the limited total RNA amount in several of the small biopsy samples, the starting isolated total RNA was used in its entirety for probe synthesis. Secondly, differences between the two gene expression analysing method could be due to different gene sequences being amplified and detected during the real-time RT-PCR and the microarray analysis.

EGFR-immunohistochemistry

In colorectal carcinoma samples, EGFR was found to be mildly and moderately expressed in all carcinoma cells. Diffuse cytoplasmatic EGFR staining was found in all carcinoma samples. In normal samples, moderate and high EGFR expression was found in some epithelial cells, but in total amount, EGFR expression was decreased compared to carcinoma samples (Figure 3). EGFR protein expression data were in correlation with EGFR mRNA expression data from both microarray and real-time RT-PCR analysis.

DISCUSSION

We compared the gene expression pattern of biopsy samples from patients with adenoma, CRC and IBD, on

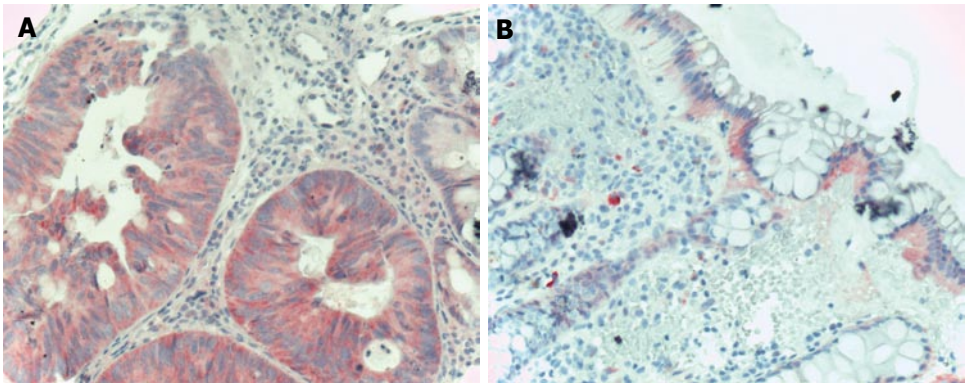

Figure 3 EGFR immunohistochemistry in CRC. **A**: Carcinomatous glands of the colon showing diffuse cytoplasmatic, moderately intensive EGFR staining (Hematoxylin co-staining × 200); **B**: Normal colonic epithelia showing mildly, moderately intensive basolateral intracytoplasmatic EGFR staining. The lower 2/3 of the crypts do not show EGFR positivity (Hematoxylin co-staining × 100).

Human Atlas Glass 1.0 microarrays containing 1081 target sequences. Biopsies were taken from disease-involved areas of the colon and surrounding disease-free colonic mucosa from the same patient. Multiple filtering methods for reduction of array-array variability origins from the spotted microarray hybridization procedure, and multiple statistical analyses were applied for finding colorectal carcinogenesis-associated genes, which can enhance the conventional histological diagnosis.

The Atlas Glass Human 1.0 microarray platform is a spotted microarray with each probe consisting of a single "long oligo" (80 mer) spotted on a glass slide. On the Atlas Glass microarray, all of the weight of detection rests on a single oligonucleotide. If cross-hybridization with an inappropriate target occurs, then the readout for that gene is incorrect. This could result in an erroneous quantification of mRNA levels[19,20] and relatively high standard deviation values. This main disadvantage of spotted opened system microarrays was apparent in our analyses: several hybridized microarrays (12 from 34) were removed from the further analysis after the quality testing, because of the problems mentioned above. Hence, comparison results from analysis of different microarray types, besides the facts mentioned above, requires due foresight, because of the different signal detection techniques, various sample amounts, sample types, sample processing and storage, and different experimental controls. The results are strongly affected by choice of biopsy samples or surgically resected tissue as a starting material, heterogeneous tissue samples or homogeneous cells such as laser microdissected cells. Our concept was that biopsies taken from patients during endoscopical examination with minimal intervention, are the most suitable samples for identifying early diagnostic target molecules.

CRC cases are characterized by upregulated genes in the DNA replication, cell cycle, extracellular matrix remodeling, transcription regulation, oncogenesis and growth factor related cell proliferation cell function groups; and downregulated genes in the DNA repair, tumor suppression and apoptosis cell function groups, while adenoma cases showed altered gene expression data in apoptosis, growth factors, receptors and their signal transduction, oncogenesis and tumor suppression functional groups. Despite the differences in samples and microarray types, there are genes, which were found differently expressed in our study that were also found commonly with other research groups. This fact emphasizes the importance of these CRC-related genes. Similarly to our findings, overexpression of several oncogenes (v-jun[20], BRCA2[8], growth factor genes (VEGF[22], HGF[23], TGFβ[5,9,24,25]), DNA replication and repair genes (TOP2A[8,26], tankyrase[9]), signal transduction gene (ephrin B2[27]), cell cycle genes (cell growth regulatory with zinc finger domain[8]) and transcription factors (several interferon regulatory factors[8]) was previously found by others using microarray analysis in gastrointestinal cancers.

Over- and underexpression of several apoptosis-, proliferation- and cell survival-related genes were observed in our microarray analysis (Table 1). These cell processes and their balance play a critical role in carcinogenesis and tumor progression. Functional analysis and visualization of biological association networks, especially pathways involved in apoptosis and cell survival were done using Pathway Assist 2.53 software. Figure 4 shows a graphical overview illustrating the role of the most important upregulated apoptosis-related genes. We found differently expressed genes in our study that are involved in or affected by not only one, but four different apoptotic pathways which are known in mammalian cells. These genes include TNFRSF10c (tumor necrosis factor receptor superfamily, member 10c) so called TRAILR3, TRAF6 (TNF receptor-associated factor 6), TNFRSF6 (Fas) and caspase 4 (CASP4) genes, downregulated apoptotic BIK (Bcl-2 interacting killer) and PP2A (protein phosphatase 2A). Two other genes that showed increased mRNA level in CRC, and were verified by real-time RT-PCR and/or immunohistochemistry, DNA topoisomerase I (TOP1) and EGFR (epidermal growth factor receptor), have a possible role in cell processes leading to CRC. Overexpression of EGFR correlates with CRC progression and metastatic potential[28]. EGFR activation can promote proliferation and maintain survival via MAPK (Ras/JNK), Ras/ERK and JAK/STAT signalling pathways. Amplification of receptor signaling by means of overexpression may promote tumor growth and resistance to apoptosis. Human TOP1 associated with intensive cell division and DNA replication occurring in the malignant cells.

We also aimed to find genes that can enhance the molecular classification of CRC and its precancerous stages. Several genes from different functional groups were found to be overexpressed in both adenoma and CRC samples compared to adjacent normal mucosa, and the degree of upregulation of these genes follows the normal-adenoma-

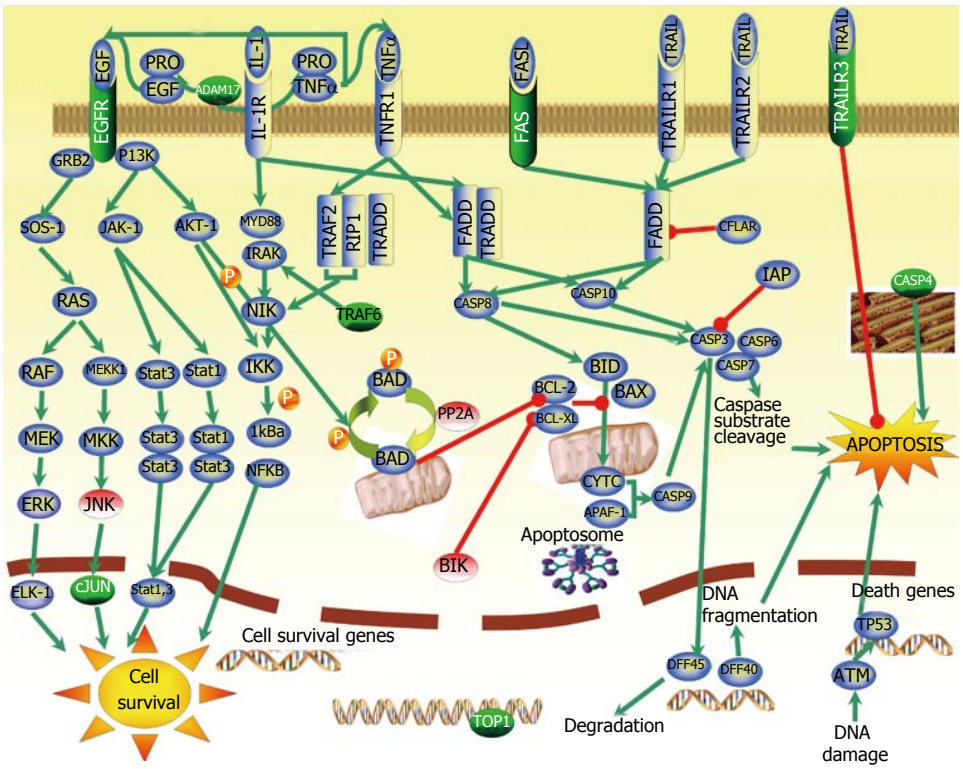

Figure 4 The main apoptotic and cell survival pathways identified in CRC gene expression study. Genes marked with green were found to be overexpressed, while genes marked with red were found to be underexpressed in our microarray analysis. Genes in blue are previously described as apoptotic and cell survival pathways-related genes. Green arrows refer to the positive regulation, while reds mean negative regulation (inhibition).

CRC sequence. Genes found to be important for this classification include cell survival promoting molecules (TRAF6, Grb10, ELF2, TACE), DNA synthesis and cell cycle involved molecules (RanGAP1, replication protein A1) and CFTR chloride channel. CFTR (cystic fibrosis transmembrane conductance regulator) is the most important chloride channel in the luminal membrane of the colon. A central role has been suggested for CFTR in coordinating electrolyte transport by changing absorption into secretion in colon carcinogenesis[29]. Recent studies demonstrate an enhanced cAMP-activated Cl^- secretion in the hyperproliferative colonic mucosa that is caused by elevated CFTR expression[30].

IBD cases are featured by the gene expression changes of immune regulation, transport and growth factor-related cell proliferation in our study. In correlation with our findings, elevated chemokine (small inducible cytokine A4, interleukin-1 receptor antagonist) and matrix metalloprotease (MMP-3, MMP-12) mRNA levels were detected in IBD compared to normal mucosa by microarray analysis[16]. Small inducible protein A4 is highly expressed in IBD according to the degree of inflammation[31]. IL-1 receptor antagonists inhibit the activity of IL1 and modulate a variety of IL1-related immune and inflammatory responses. Programmed expression of MMPs is involved in tissue remodeling during inflammation, moreover MMP-12 may play a role in macrophage movement and epithelial cell shedding[32]. Several other growth factors and chemo attractants were found overexpressed in our microarray analyses. Secretion of GM-CSF2 (macrophage-granulocyte colony stimulating factor 2) is increased in mucosal lesions in IBD. B cell growth factor 1 released by T cells after antigen stimulation, and supports the clonal proliferation of B cells. CXCL13 B cell chemoattractant is mostly produced by the monocyte/macrophage lineage in UC[33].

Discrimination of colonic diseases according to the gene expression markers

Discriminant analysis shows the genes which can help us to classify an unknown sample into one of the groups of observed diseases, considering their expression levels. According to the expression changes of the following four genes the three colonic disease groups can be significantly distinguished: HSF1, calgranulin A, TNFRSF10c (TRAILR3) and bystin-like.

HSF1 is a heat-shock transcription factor, that was found to be upregulated in CRC compared to normal mucosa in both our and other research groups' gene expression studies[34]. In adenoma and IBD cases it showed lower expression level compared to the CRC samples. Induction of HSF1 gene expression could activate the HSF1 heat shock stress signal pathway in sporadic CRC. The heat shock stress signaling pathway is highly involved in carcinogenesis since heat shock proteins are responsible for maintaining the conformation, stability and function of key oncogenic client proteins involved in signal transduction pathways leading to proliferation, cell cycle progression and apoptosis, as well as other features of the malignant phenotype such as invasion, angiogenesis and metastasis[35-37].

Calgranulin A (S100A8) is a calcium-binding protein that showed higher expression in the IBD sample group, than in CRC and adenoma cases. S100 proteins are involved in the regulation of a number of cellular processes such as cell cycle progression, differentiation and immune response[38].

Significantly increased TRAILR3 level was found in

CRC samples, but not in IBD and adenoma cases. This receptor inhibits the TRAIL-induced apoptosis via binding TRAIL ligand which in this case cannot interact with the pro-apoptotic, death domain containing other TRAIL receptors[39,40].

Interestingly, significant bystin-like 7-fold mRNA overexpression was found in IBD samples in our microarray analysis. Suzuki *et al*[41] identified a cytoplasmic protein, named bystin, that directly binds trophinin and tastin cell adhesion molecules that are involved in the process of the embryo implantation. A role for bystin-like protein in inflammation has not been described.

The factor analysis gives information about the functional gene groups which can differentiate the observed diseases according to their different expression levels. Five of the seven genes (HSF1, TRAILR3, stem cell growth factor, interleukin-7 receptor and pleiotrophin) with the most considerable explorative variance value (belong to Factor 1) were mentioned previously in the scientific literature as cell proliferation and cancer related genes (HSF1[34-37], TRAILR3[39,40], stem cell growth factor[42], interleukin-7 receptor[43] and pleiotrophin[44,45]).

Twenty two colonic biopsy samples were clustered in correlation with the conventional histopathological diagnoses. Excluding one case, all adenoma cases belong to a significantly distinct cluster. The cluster of severely inflammed IBD cases contained one colorectal adenocarcinoma case, as it showed a similar gene expression pattern. This fact can refer that colorectal cancer can develop on the basis of chronic inflammation and some types of adenomas.

In summary, we can say that the overall mid-size glass arrays are suitable for identification of disease-specific genes which are considered as gene expression markers. Detection of the mRNA expression levels of marker gene panels gives an opportunity for classification of colonic samples, even in the case of small biopsy specimens. The limited starting RNA amount arising from the small sample size makes microarray analysis difficult. The standardization of the opened manual microarray systems is more difficult; however, automatization of this technique would further improve the efficacy, reproducibility and quality of microarray analysis.

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RAPID COMMUNICATION

Colorectal cancer screening by non-invasive metabolic biomarker fecal tumor M2-PK

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Abstract

AIM: To evaluate the utility of the innovative fecal tumor M2-Pyruvate kinase (M2-PK) test in our daily clinical routine, as a marker for the pre-selection of patients who should subsequently undergo colonoscopy for the diagnosis or exclusion of colorectal cancer.

METHODS: Fecal tumor M2-PK was measured in stool samples of 96 study participants (33 patients with colorectal cancer, 21 patients with rectal carcinoma and 42 controls) who all underwent total colonoscopy.

RESULTS: In 39 of 42 individuals in the control group, fecal tumor M2-PK was below 4.0 kU/L (93% specificity). Colorectal tumors were accompanied by a highly significant increase ($P < 0.001$) in fecal tumor M2-PK levels (median: colon carcinoma, 23.1 kU/L; rectal carcinoma, 6.9 kU/L; colorectal carcinoma, 14.7 kU/L), which correlated with Duke's staging and T-classification. The overall sensitivity was 78% for colorectal cancer, increasing from 60% for stage T1 to 100% for stage T4 and from 60% for Duke's A to 90% for Duke's D tumors.

CONCLUSION: Fecal tumor M2-PK is an appropriately sensitive tool to pre-select those patients requiring colonoscopy for the further diagnostic confirmation or exclusion of colorectal cancer.

Key words: Tumor M2-Pyruvate kinase; Pyruvate kinase type M2; Colon cancer; Rectal cancer; Adenoma; Feces; Cancer screening

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INTRODUCTION

In Germany, about 70 000 people are diagnosed with colorectal cancer each year[1]. This figure is about 1 million worldwide, with approximately 528 000 deaths from colorectal cancer each year[2]. The gold standard for the early detection of colorectal cancer is colonoscopy. However, the acceptance of this costly and invasive method is low. Only 1.7% of people entitled to colonoscopy under the German national colorectal cancer screening program actually undergo the procedure[3].

In order to increase the participation in colorectal cancer screening programs, an easy, fast and economical initial screening method, with good patient compliance, is absolutely necessary. This allows identification of those patients most likely to have colorectal cancer, who require further investigation by colonoscopy.

The guaiac-based fecal occult blood test (FOBT), which is based on the premise that polyps and cancer bleed more than normal mucosa[4], is currently the most commonly used test for colorectal cancer screening. Guaiac-based FOBTs have been investigated in a number of large studies and shown to reduce mortality by about 15%-33% in screened populations[5,6]. However, they have limited sensitivity. For example, Lieberman *et al*[7] and Koss *et al*[8] found their sensitivity was less than 30% for colorectal cancer and less than 15% for advanced adenomas. Newer immunological FOBTs showed higher sensitivities[9,10] with the advantage of no dietary restrictions. In most studies with immunological FOBTs to date, however, colonoscopy has been performed only in FOBT-positive cases. Non-bleeding colorectal tumors and those not consistently discharging sufficient blood into the gut lumen are not detected by either guaiac or immunological FOBTs.

Recently a new screening test for the early detection of adenomas and colorectal tumors has been described. The tumor M2-Pyruvate kinase (M2-PK) stool test is based on the measurement of a key enzyme involved in tumor metabolism[8,11-14].

Tumor M2-PK is the dimeric form of the glycolytic pyruvate kinase isoenzyme type M2[15]. The enzyme catalyzes the last reaction step within the glycolytic

sequence from phosphoenolpyruvate (PEP) to lactate and is responsible for net ATP production within this pathway. Enzymatic characterization of a wide range of different tumors revealed that tumorigenesis is accompanied by an increase in total pyruvate kinase v-max activities. There is also a shift towards the expression of the pyruvate kinase isoenzyme type M2 (M2-PK) and away from the tissue-specific isoenzymes (L-PK in liver and kidney, M1-PK in muscle and brain and R-PK in erythrocytes)[16-18]. The increased expression of M2-PK is under the control of ras, and the transcription factors SP1 and HIF-1. Ras and HIF-1 are both consistently altered in gastrointestinal tumors[19-22]. M2-PK can occur in a tetrameric form which is characterized by a high affinity to its substrate PEP and in a dimeric form with a low PEP affinity. The tetramer: dimer ratio of M2-PK determines the proportion of glucose carbons used for glycolytic energy production (tetrameric form) or channeled into synthetic processes (dimeric form). In tumor cells, M2-PK is mainly found in the dimeric form (tumor M2-PK) due to direct interaction with various oncoproteins, i.e. $pp60^{v\text{-}src}$ kinase and HPV-16 E7[15,21,23]. Tumor M2-PK is released into the blood, and in the case of adenomas and tumors in the lower gastrointestinal tract also into the stool of patients. An increase in tumor M2-PK in EDTA plasma samples is found in gastrointestinal cancers, as well as a wide range of other tumors such as lung, renal, breast and cervical cancer. The EDTA plasma test is highly suitable for patient monitoring[24-30].

The fecal tumor M2-PK test has been described as a promising new screening tool for adenomas and colorectal cancer[8,11-14]. Therefore, the aim of our study was to evaluate the utility of the tumor M2-PK test in our own daily clinical routine as a marker for the pre-selection of patients requiring subsequent diagnostic colonoscopy.

MATERIALS AND METHODS

Patients

Our study consisted of 96 participants who underwent complete colonoscopy. The control group consisted of 42 healthy individuals (15 male and 27 female; median age: 58 years; range: 25-79 years) without any findings at colonoscopy, who were participating in the national screening colonoscopy program provided by the German health insurance system. All screening colonoscopies were conducted between September 2005 and April 2006 in a primary care gastroenterology and hepatology medical center. Healthy individuals were included in the control group.

The 54 participants with colorectal cancer underwent diagnostic colonoscopy at the Offenbach Municipal Hospital between January 2003 and April 2006. Rectal carcinomas were diagnosed in 21 patients (15 male and 6 female; median age: 70 years; range: 52-84 years). Colonic adenocarcinomas were diagnosed in 33 patients (24 male and 9 female; median age: 70 years; range: 43-84 years).

All participants received a stool sample collection pot and were instructed to collect a single stool sample (naturally produced, walnut sized) one day prior to the laxative administration in preparation for colonoscopy. No special diet was recommended. Paper collecting devices were used to avoid stool contact with water in the toilet bowl. Stool samples were initially stored at room temperature by the participants until the day of colonoscopy. Thereafter, these pre-colonoscopy stool samples were stored at -20℃ at the medical center or hospital until analyzed for tumor M2-PK.

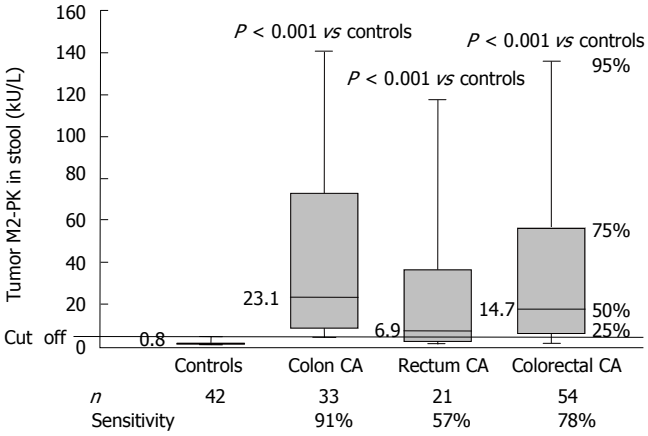

Figure 1 Tumor M2-PK levels in stool samples of healthy control individuals and patients with colon or rectal cancer.

Measurement of fecal tumor M2-PK concentrations

Fecal tumor M2-PK concentrations were determined using a commercially available sandwich ELISA based on two different monoclonal antibodies which specifically recognize the dimeric form of M2-PK (ScheBo® · Biotech AG, Giessen, Germany). A positive test result was defined as > 4.0 kU/L, as indicated by the manufacturer.

Statistical analysis

Since the data were skewed to the right, the statistical analysis was conducted using the Kruskall-Wallis ANOVA test (Statistica, StatSoft® Inc., Tulsa, USA).

RESULTS

This study evaluated 54 patients with colorectal cancer and 42 healthy controls with no indication of gastrointestinal diseases at colonoscopy. In the control group, fecal tumor M2-PK levels were below 4.0 kU/L in 39 of the 42 subjects (median: 0.8 kU/L), resulting in 93% specificity (Figure 1). In two of the three control samples which were above the cut-off value only a slight increase of tumor M2-PK (4.4 kU/L and 5.3 kU/L) was measured.

Colorectal tumors were accompanied by a highly significant increase in fecal tumor M2-PK levels. The median value was 23.1 kU/L for colon carcinoma ($P < 0.001$), 6.9 kU/L for rectal carcinoma ($P < 0.001$) and 14.7 kU/L ($P < 0.001$) for colorectal carcinoma when both groups were combined.

At a cut-off level of 4.0 kU/L, the sensitivity was 91% for colon carcinoma, 57% for rectal carcinoma and 78% when both groups were combined. Both T classification and Duke's staging of the colorectal tumors revealed a

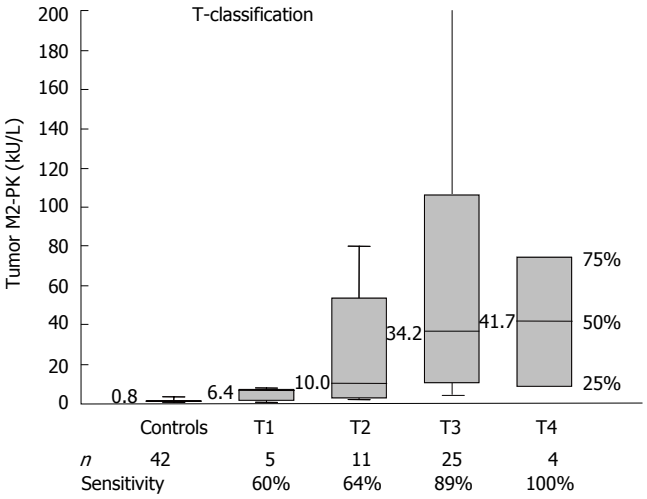

Figure 2 Correlation between fecal tumor M2-PK levels and TNM staging.

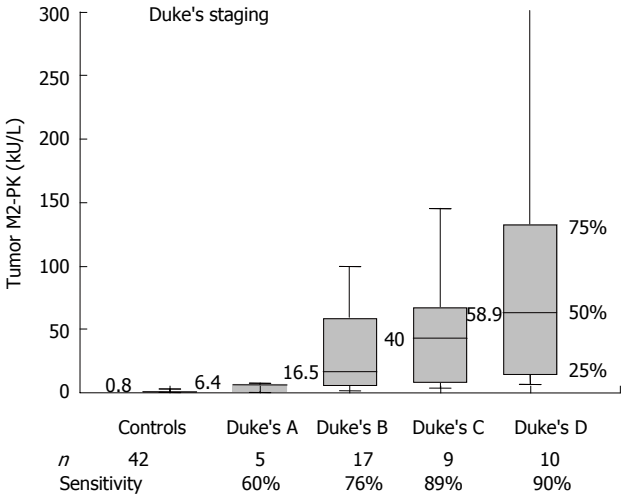

Figure 3 Correlation between fecal tumor M2-PK and Duke's staging.

Table 1 Correlation between fecal tumor M2-PK levels and TNM classification or duke's staging

Classification	*n*	Median (kU/L)	Mean (kU/L)	SE (kU/L)	Range (kU/L)
Controls	42	0.8	1.5	0.4	0.1-17.3
T1	5	6.4	4.5	1.6	0.3-7.7
T2	11	10.0	30.5	10.1	1.5-100
T3	25	34.2	106.3	35.2	1.7-620
T4	4	41.7	41.4	19.2	6.6-76
Duke's A	5	6.4	4.5	1.6	0.3-7.7
Duke's B	17	16.5	63.5	34.7	0.2-604
Duke's C	9	40.0	50.0	17.8	1.9-176
Duke's D	10	58.9	138.5	65.3	4.5-620

strong correlation between fecal tumor M2-PK levels and staging. The sensitivities increased from 60% for stage T1 to 100 % for stage T4 and from 60% for Duke´s A to 90% for Duke's D (Figures 2 and 3; Table 1).

DISCUSSION

Tumor M2-PK is the synonym for the dimeric form of the glycolytic pyruvate kinase isoenzyme type M2[15]. M2-PK is the pyruvate kinase isoenzyme which is characteristic of all proliferating cells and can occur in a tetrameric form as well as a dimeric form. Previous studies describe that tumor M2-PK is released into the stool of patients with adenomas and colorectal tumors and can easily be quantified with a commercially available sandwich ELISA[8,11-14].

In order to evaluate whether tumor M2-PK is a practical tool for the pre-selection of patients with colorectal cancer in our daily routine, we measured fecal tumor M2-PK in a cohort of 96 individuals. All 42 healthy control individuals, 33 patients with colon carcinoma and 21 patients with rectal carcinoma underwent total colonoscopy in order to confirm or exclude colorectal cancer. Our study revealed a highly significant increase ($P < 0.001$) in tumor M2-PK in the stool samples of those patients with colorectal cancer, whereby fecal tumor M2-PK values correlated well with Duke's staging and T-classification (Figures 1-3; Table 1). Even stage T1 or Duke's A showed 60% sensitivity, increasing to 100% in stage T4 and to 90% in Duke's D tumors.

At a cut-off value of 4.0 kU/L, our overall sensitivity for colorectal carcinoma was 78 %. These data correspond well with the results of Hardt *et al*[13] who reported a sensitivity of 78% in 60 colorectal cancer patients, and those of Naumann *et al*[31] who found a sensitivity of 85.2% in a cohort of 27 colorectal cancer patients. A higher sensitivity was reported by McLoughlin *et al*[14] (92% in 25 colorectal cancer patients and 67% in 30 patients with adenomas) and by Koss *et al*[8] (92.3 % in 26 colorectal cancer patients and 60% for adenomas > 1 cm in ten patients).

The most commonly used fecal test in current screening programs is the guaiac-based FOBT[32,33]. Liebermann *et al*[7] and Koss *et al*[8] have reported an overall sensitivity for guaiac-based FOBTs of less than 30% for colorectal cancer and less than 15% for advanced adenomas. Results with newer, immunological FOBTs showed higher sensitivities than guaiac-based FOBTs for colorectal cancers[9,10] but in most studies colonoscopy was performed only in FOBT-positive cases.

The overall sensitivity of tumor M2-PK is increased if a higher proportion of late stage patients are included, but this is also true for FOBTs[10]. Nevertheless, McLoughlin *et al*[14] reported a sensitivity of 67% for adenomas with the fecal tumor M2-PK test. Similarly, Koss *et al*[8] found a sensitivity of 60% for adenomas > 1 cm.

In a head-to-head comparison of fecal tumor M2-PK and the commonly used guaiac-based FOBT, Koss *et al*[8] demonstrated a sensitivity for colorectal cancer of 92.3% for fecal tumor M2-PK and 20% for FOBT. No comparative study of fecal tumor M2-PK and immunochemical FOBTs is currently available.

The high sensitivity of the tumor M2-PK test is due to its ability to detect bleeding and non-bleeding tumors. From a practical viewpoint, the use of a single random formed stool sample for tumor M2-PK analysis, without

requiring dietary restrictions, might be of greater patient convenience compared with the need to collect stool on three consecutive days for the guaiac FOBT.

In our study, the control group consisted of individuals without any signs of gastrointestinal diseases at colonoscopy. The median tumor M2-PK value in this group was 0.8 kU/L. In 39 of 42 subjects, tumor M2-PK levels were below the cut-off value; in two further control samples tumor M2-PK levels were only slightly increased (4.4, 5.3 kU/L). The resulting specificity at a cut off value of 4.0 kU/L is 93%, which is in general accordance with the studies of Hardt *et al*[13], Koss *et al*[8] and McLoughlin *et al*[14] who report specificities between 78% and 98%. Naumann *et al*[31] found increased fecal tumor M2-PK levels in cases of active Crohn's disease and ulcerative colitis in which increased cell proliferation is expected. In addition, patients with inflammatory bowel disease have an increased risk of developing colorectal cancer, probably linked to frequent cycles of damage and regeneration of the colonic mucosa associated with flares of active disease.

Another new approach for pre-selective colorectal cancer screening is the determination of mutated oncogenes and anti-oncogenes[22,34-38]. These tests have the advantage of very high specificities. However, due to high genetic heterogeneity within colorectal cancers, a panel of different targets (k-ras, p53, APC genes, as well as microsatellite instability marker) must be measured in order to reach acceptable sensitivity which makes the test extensive and expensive. Using a 21-target multipanel, sensitivities between 44% and 91% and specificities between 93% and 100% are described[36-38]. Furthermore, to assure the stability of DNA within the stool, samples have to be frozen at -80℃ within 12 h after defecation. Fecal tumor M2-PK is stable for 48 h at room temperature and for up to one year when frozen at -20℃ (manufacturer's data sheet), which makes it practical for routine use. In addition, the tumor M2-PK test could be conducted in virtually all hospital and private diagnostic laboratories because it can either be run manually combined with an ELISA plate reader or automated using existing commercially available equipment.

Overall, our results are in general agreement with previous studies which have demonstrated that fecal tumor M2-PK is an appropriate tool to achieve a sensitive pre-selection by identifying those patients with the greatest need to undergo diagnostic colonoscopy to confirm or exclude colorectal cancer.

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RAPID COMMUNICATION

Steroids reduce local inflammatory mediator secretion and mucosal permeability in collagenous colitis patients

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Abstract

AIM: To study the effect of oral steroids upon clinical response and rectal mucosa secretion of eosinophil cationic protein (ECP), myeloperoxidase (MPO), basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF) and albumin in patients with collagenous colitis (CC).

METHODS: A segmental perfusion technique was used to collect perfusates from rectum of CC patients once before and twice (one and four weeks) after the start of steroid treatment. Clinical data was monitored and ECP, MPO, bFGF, VEGF and albumin concentrations were analyzed by immunochemical methods in perfusates and in serum.

RESULTS: Steroids reduced the number of bowel movements by more than five times within one week and all patients reported improved subjective well-being at wk 1 and 4. At the same time, the median concentrations of ECP, bFGF, VEGF and albumin in rectal perfusates decreased significantly. MPO values were above the detection limit in only 3 patients before treatment and in none during treatment. VEGF, bFGF, ECP and albumin concentrations correlated with each other with the exception of ECP and albumin. A decrease of serum ECP and VEGF concentrations was also seen even if the overtime reduction was not significant.

CONCLUSION: Oral steroid treatment in CC patients induced a simultaneous reduction of bowel movements and rectal release of ECP, bFGF, VEGF and albumin, suggesting that these polypeptides and increased mucosal permeability are important components of the pathophysiology in collagenous colitis.

Key words: Collagenous colitis; Inflammatory mediator; Steroid treatment; Eosinophil cationic protein; Basic fibroblast growth factor; Vascular endothelial growth factor

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INTRODUCTION

Collagenous colitis (CC) is characterized by watery diarrhea and an increased subepithelial collagen layer with infiltration of inflammatory cells in the surface epithelium and lamina propria of the colon[1]. The thickened collagen in CC is an amorphous eosinophilic band located beneath the intercryptal epithelial surface and is best visualized using special collagen staining, such as Masson´s trichrome[1]. The collagen composition of the basal membrane of colonic mucosa in CC patients is identical to that of normal individuals[1]. In contrast, the subepithelial collagen layer differs in CC patients and consists of collagen type I and Ⅲ or Ⅵ[2-4].

Intestinal inflammatory cells present in CC are lymphocytes, plasma cells, often eosinophil and sometimes neutrophil granulocytes. In the epithelium, lymphocytes are most frequent and dominated by $CD8^+$ T-cells[5]. In the lamina propria, the inflammatory cell infiltration includes plasma cells, eosinophils, macrophages and mast cells, but rarely neutrophils. Unlike epithelial lymphocytes, the lymphocytes in the lamina propria are mainly composed of $CD4^+$ T-cells[5].

Eosinophils are one of the main components of the inflammatory cell population in colonic mucosa of CC patients[1]. Eosinophils produce not only cytotoxic granule proteins, such as eosinophil cationic protein (ECP), major

basic protein (MBP) or eosinophil peroxidase (EPO), but also a wide range of polypeptides, such as transforming growth factor-β1 (TGF-β1), vascular endothelial growth factor (VEGF), *etc*[6-10]. Eosinophilopoietic cytokines, such as granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin-5 (IL-5), activate eosinophils by inhibiting apoptosis and prolonging the life span of eosinophils[7]. Eosinophils interact with fibroblasts and contribute to fibrosis in inflammatory diseases like asthma and inflammatory bowel disease (IBD). Activated fibroblasts/myofibroblasts are regarded as the major contributors of collagen accumulation in the intestinal wall of IBD[11,12] including CC patients[13].

In asthma, basic fibroblast growth factor (bFGF), separately or in combination with other growth factors, perpetuate chronic inflammation and triggers fibrosis leading to airway tissue remodeling[14] including excessive extra cellular matrix deposition in the subepithelial region. The role of bFGF, as one contributing factor in tissue remodeling, is supported by perfusion studies in ulcerative colitis (UC)[15] and CC[16] patients, where the secretion of bFGF was increased.

VEGF is a pleiotropic polypeptide, which has physiological and pathophysiological actions on the extra cellular matrix and may enhance fibrosis through induction of connective tissue growth factor (CTGF)[17]. A recent study on the pathophysiological role of VEGF in CC showed that VEGF, by changing the local matrix metalloproteinase -1 and tissue inhibitor metalloproteinase-1 (MMP-1/TIMP-1) balance, causes the accumulation of immature subepithelial ECM in CC[18].

Glucocorticosteroids have several anti-inflammatory effects, including the ability to block the recruitment of proinflammatory cells like eosinophils, downregulation of the secretion of proinflammatory cytokines, enhancement of apoptosis in eosinophils[19-21] and engulfment of apoptotic eosinophils by phagocytic cells[7]. In asthma, prolonged treatment with inhaled corticosteroids reverses tissue remodeling by downregulating fibroblast-functions[14,20,22]. In our previous perfusion studies, we found increased colorectal concentrations of ECP[23], bFGF[16], VEGF[24] and the permeability marker albumin in CC patients compared with control patients. We put forward the hypothesis that these mediators enhanced chronic inflammation and fibrosis, leading to pathological accumulation of subepithelial collagen. Oral steroid treatment is effective for inducing remission in CC patients[25,26] but the mechanisms leading to relief of symptoms are not fully known. The aim of this study was to elucidate the relationship between steroid treatment and rectal mucosa secretion of ECP, MPO, bFGF, VEGF and albumin in CC patients.

MATERIALS AND METHODS

Patients

Twelve patients (10 women, 2 men; mean age 52 years; range 34-66 years) who fulfilled the diagnostic criteria of CC[1] were recruited between November 2000 and November 2002 at the Department of Medicine, University Hospital, Uppsala, Sweden.

Ethics

Committee of the Medical faculty at Uppsala University approved the study and informed consent was obtained from all participants. No patient had anti- or pro-inflammatory drug treatment during the investigation period, but one patient (number eight) was taking indomethacin (Indomee^{R}, Merck Sharp and Dohme, Stockholm, Sweden) up until one week before the first perfusion. Patient number seven also had celiac disease and lactose intolerance, while patients five and eight suffered from hypothyroidism. Patients eleven and twelve were smokers.

Design of study

Steroid treatment was initiated after the first perfusion with an oral dose of 40 mg prednisolone daily, which was reduced successively by 5 mg/wk. Three rectal perfusions were fulfilled per patient, one before and two after the start of steroid treatment, at completed weeks one and four respectively. Serum samples were also collected on those occasions. Clinical data were recorded at each perfusion as the patients estimated their average daily number of loose stools and degree of general well-being during the week preceding each perfusion.

Perfusion technique

A colonoscope-based segmental perfusion technique[23] was used to obtain perfusion fluid from the rectum. Sodium phosphate (Phosphoral^{R}, Ferring, Limhamn, Sweden) was used for bowel preparation. The rectal perfusions were carried out with the patients lying in a supine position and started with an endoscopic examination of the rectum and sigmoid colon. The segment, positioned at the end of the flexible endoscope, was established by inflating air into two balloons delimiting the segment in the rectum. The perfusion procedures were carried out for one hour[23] and the perfusates were collected in three portions at 20 min intervals in tubes immersed in ice. Intravenous injections of the sedative diazepam (Stesolid^{R}, Alpharma, Stockholm, Sweden) were given at a median dose of 7.5 mg (range: 5-15) and the analgesic meperidine (Petidin^{R}, Ipex, Stockholm, Sweden) at a median dose of 25 mg (range: 25-50).

Perfusion buffer, perfusates and serum samples

A buffer was used, consisting of 120 mmol/L NaCl, 5.4 mmol/L KCl, 2 mmol/L Na_2HPO_4, 10 mmol/L glucose, 35 mmol/L mannitol, and 1 g/L polyethylene glycol (PEG; MW 4 kDa) at pH of 8.20-8.48 and osmolarity of 290 mosm/L. The perfusion fluid was kept at 37℃ and then infused at a speed of 3 mL/min for 60 min. By adding ten milliliters of aprotinin (Trasylol^{R} Bayer 1.0 × 10^7 kIU/L) to every liter of perfusion buffer, proteolytic activity was inhibited. Phenol red solution (50 mg/L in physiological saline) was intermittently infused (5 mL every 20 min) above the proximal balloon to check that the perfusion segment was closed and that there was no contamination of the perfusion fluid by intestinal contents located proximally. All perfusate samples were collected on ice and frozen in aliquots of 2 mL at -70℃. The protease inhibitor, phenylmethylsulphonyl fluoride (PMSF; Sigma

Table 1 The clinical data and the individual concentrations of ECP, MPO, bFGF, VEGF and albumin in rectal perfusion fluid of CC patients before and after one and four weeks of treatment with steroids

Demography			Bowel movements per day			Mediators analyzed in Perfusion fluid														
						ECP (μg/L)			MPO (μg/L)			bFGF (pg/mL)			VEGF (pg/mL)			Albumin (mg/L)		
Patients	Age (yr)	Gender				wk														
			0	1	4	0	1	4	0	1	4	0	1	4	0	1	4	0	1	4
1	34	Female	3-4	0-1	0-1	3.7	< 2	< 2	29.1	14.9	< 8	14.03	18.26	0.65	299.7	92.8	41	20.3	44.6	< 2.1
2	57	Female	4	0-1	0-1	2.6	< 2	< 2	12.5	< 8	< 8	7.85	7.11	0.37	54.7	97	40.2	95.8	57.7	< 2.1
3	59	Female	10-12	0-1	0-1	3.6	7	< 2	< 8	< 8	< 8	1.21	4.1	< 0.25	113.1	146.7	81.1	20.3	10.7	3.4
4	43	Female	4-6	3-4	0-1	< 2	< 2	< 2	< 8	< 8	< 8	0.72	0.41	< 0.25	28.4	22.9	40.2	2.8	10.9	17.5
5	62	Female	3-4	0-1	0-1	< 2	< 2	< 2	< 8	< 8	11.3	0.35	< 0.25	0.29	66.2	46.6	53.1	2.7	10.7	5.5
7	66	Female	4-5	0-1	0-1	< 2	< 2	< 2	< 8	< 8	< 8	< 0.25	< 0.25	< 0.25	58.8	19.1	22.2	3.9	6.5	3.2
8	59	Female	5-10	2	0-1	6.5	< 2	< 2	< 8	< 8	< 8	5.28	< 0.25	0.35	86.1	37.8	58.8	17.1	3.1	2.3
9	64	Female	3	0-1	0-1	< 2	< 2	< 2	< 8	< 8	< 8	< 0.25	< 0.25	< 0.25	17.6	9.6	12.3	5	< 2.1	7
10	39	Male	7-10	2-3	0-1	< 2	< 2	< 2	< 8	< 8	< 8	2.7	1.21	< 0.25	100.4	94.5	90.3	43	13.7	11.7
11	34	Female	5-6	2	0-1	3.1	< 2	< 2	18.3	< 8	< 8	11.99	2.35	< 0.25	153.6	84.4	80.2	116	22.1	10.7
12	53	Male	6-7	2-3	0-1	2.3	< 2	< 2	< 8	< 8	< 8	12.29	0.91	0.61	95.3	48.2	29.1	21.5	5.2	< 2.1

Chemical Co., St Louis, MO, USA), was added to a final concentration of 2 mmol/L immediately before analysis to counteract the effect of even small amounts of proteases in the perfusion fluid. Serum samples were drawn simultaneously with the perfusions being performed and frozen at -70℃ for later analysis.

Quantification of ECP, MPO and albumin

The concentrations of ECP and MPO in perfusion fluids and serum samples were analyzed by means of a specific RIA (Pharmacia & Upjohn, Diagnostic AB, Uppsala, Sweden). The inter- and intra-assay coefficients of variations were < 10% for both tests. The lower detection limits of ECP and MPO in perfusion fluid were 2 and 8 μg/L, respectively. The reference intervals for ECP and MPO in serum were 2.3-16 μg/L and 170-478 μg/L, respectively. Albumin in perfusion fluid was analyzed by rate nephelometry on a Beckman Array protein system (Beckman Instruments, Brea CA), according to the recommendations of the manufacturer. The lower detection limit in perfusion fluids of albumin was 2.14 mg/L. Analysis of albumin in serum was performed by a photometric assay (Boehringer Manheim, Mannheim, Germany) and the reference intervals were 40-51 g/L (age < 50 years) and 37-48 g/L (age > 50 years). The concentration of phenol red was assessed, after alkalinization of the perfusate samples to pH 11, by a spectrophotometric method at 520 nm.

Quantification of bFGF and VEGF

ELISA technique was used to measure bFGF (Human bFGF, Quantikine High Sensitivity, R&D Systems, Minneapolis, MN, USA) and VEGF (DVE00, R&D Systems, Minneapolis, MN, USA) as recommended by the manufacturer. The bFGF and VEGF concentrations in the samples were determined by comparing the optical density of the samples with the standard curve. The lowest detectable value for bFGF was 0.25 pg/mL and for VEGF 9 pg/mL in perfusion fluid. Reference intervals of S-bFGF were < 4.0 ng/L (males) and < 10.8 ng/L (females). The reference interval of S-VEGF was < 500 ng/L.

Statistical analysis

The Wilcoxon signed rank test for two variables was used to compare the differences between wk 0, 1 and 4, while the statistical analysis of changes over time in the perfusate and serum concentrations of ECP, MPO, bFGF, VEGF and albumin, was done by Friedman's ANOVA. Spearman rank correlation test was applied to study co-variation between each mediator and the frequency of bowel movements per day. All statistical calculations were performed on a Macintosh computer by means of a statistical package, Stat View 4.51 (Abacus, Concepta Inc.). If concentrations of the mediator proteins and albumin were below the detection limit of the assay, the value of the detection limit was used in the calculations. $P < 0.05$ was considered significant.

RESULTS

Perfusions and clinical outcome

Out of 12 CC patients studied, 11 successfully completed steroid treatment and all three perfusions according to the protocol. The excluded patients stopped taking steroids because of side effects. The number of bowel movements per day declined from median 5 before start of treatment, to 0-1 after one and 0-1 after four weeks, respectively. These differences were significant ($P < 0.0001$) (Table 1). All patients reported that senses of general well-being were better after the start of steroid treatment, compared to the period before it.

ECP, MPO and albumin

The concentrations of ECP, MPO and albumin in perfusates are shown in Table 1. The median values of ECP concentrations in perfusates were 2.3 μg/L (range: 1.9-6.5) at wk 0, 1.9 μg/L (range: 1.9-7.0) at wk 1 and 1.9 μg/L (range: 1.9-1.9) at wk 4, respectively. A significant

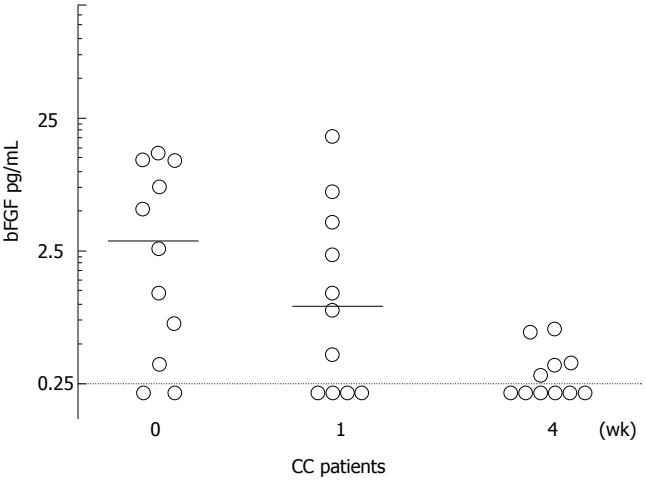

Figure 1 The individual values of basic fibroblast growth factor (bFGF) concentrations in perfusion fluids from rectum of collagenous colitis (CC) patients before and after one and four weeks of steroid treatment. The bars indicate median values. The dotted line indicates the detection limit for the assay (bFGF: 0.25 pg/mL).

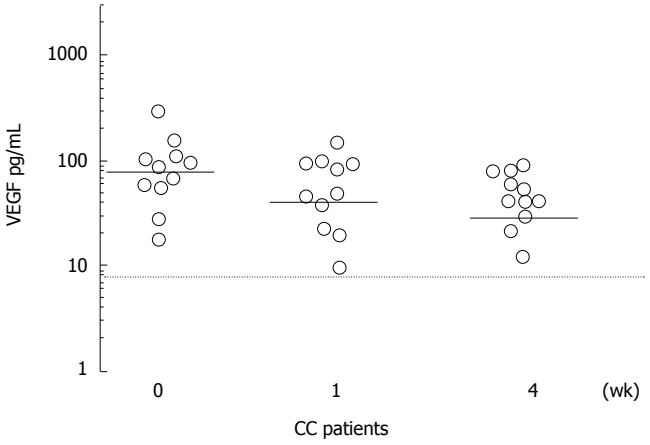

Figure 2 The individual values of vascular endothelial growth factor (VEGF) concentrations in perfusion fluids from rectum of collagenous colitis (CC) patients before and after one and four weeks of steroid treatment. The bars indicate median values. The dotted line indicates the detection limit for the assay (VEGF: 9 pg/mL).

difference was only found between wk 0 and 4 (P = 0.0277). The corresponding values for ECP in serum at wks 0, 1 and 4 were 11.3 μg/L (range: 1.9-29), 6.6 μg/L (range: 1.9-18.8) and 6.3 μg/L (range: 1.9-18.9), respectively. The decrease between wk 0 and 1 and wk 0 and 4 were significant (P = 0.0076, P = 0.045 respectively), while no difference was found when the decline of ECP concentrations in perfusate and serum over time was analyzed by the Friedman's ANOVA.

Perfusate concentrations of MPO were above the detection level in three out of 11 patients at wk 0, one at wk 1 and none at wk 4 (Table 1). Serum MPO concentrations were 432 μg/L (range: 197-649), 325 μg/L (range: 106-1805), 490 μg/L (range: 144-1190) at wk 0, 1 and 4, respectively. No difference was found between the concentrations during the study period.

The median values of albumin concentrations in perfusates were 20.3 mg/L (range: 2.7-116), 10.7 mg/L (range: 2.13-57.7) and 3.4 mg/L (range: 2.13-17.5) at wk 0, 1 and 4, respectively. The difference was significant only between wk 0 and 4 (P = 0.0329). The decline in albumin concentrations over time was not significant. The corresponding median values of serum albumin at wk 0, 1 and 4 were 42 g/L (range: 38-45), 43 g/L (range: 34-48) and 42 g/L (range: 36-46), respectively (P > 0.05). No correlation was found between the concentrations of ECP and albumin in perfusion fluids.

Analysis of bFGF

The concentrations of bFGF in perfusion fluid are presented in Table 1. The median values of bFGF concentrations in perfusion fluid were at wk 0, 1 and 4, were 2.7 pg/mL (range: 0.2-14.0), 0.9 pg/mL (range: 0.2-18.3) and 0.2 pg/mL (range: 0.2-0.7), respectively. The decline of bFGF perfusate concentrations over time was significant when analyzed by the Friedman's ANOVA (P < 0.01) (Figure 1). A correlation (P < 0.05, r = 0.702) that existed between bFGF and ECP perfusate concentrations at wk 0 disappeared at wk 1 and 4. A correlation between bFGF and albumin in perfusates remained significant throughout the study period (P < 0.05 and r = 0.709, r = 0.805, r = - 0.670 at wk 0, 1 and 4 respectively). The median values of bFGF concentrations in serum were 9.1 ng/L (range: 1.7-44), 2.9 ng/L (range: 0.9-17.6) and 3.6 ng/L (range: 0.3-20.9) at wk 0, 1 and 4, respectively, but the decline over time was not significant.

Analysis of VEGF

Table 1 shows all the individual concentrations of VEGF in perfusion fluid. The median values of VEGF concentrations in perfusates of these individuals at wk 0, 1 and 4 were 86.1 pg/mL (range: 17.6-299.7), 48.2 pg/mL (range: 9.6-146.7), and 41.0 pg/mL (range: 12.3-90.3), respectively. When the Friedman's ANOVA was used to analyze the decline of VEGF concentrations, a significant difference (P < 0.01) was seen (Figure 2). Furthermore, there was a significant correlation between VEGF and ECP at wk 0 and 1 (P < 0.05; r = 0.627 and 0.625 at wk 0 and 1 respectively) but not at wk 4. The correlation between VEGF and albumin in perfusate was significant only at wk 1 (P < 0.05; r = 0.666). Similar significant correlations were present between VEGF and bFGF concentrations at wk 0 and 1 (P < 0.01; r = 0.680 and P < 0.05; r = 0.850) but not at wk 4. The median values of VEGF serum concentrations at wk 0, 1 and 4 were 842 ng/L (range: 622-1517), 775 ng/L (range: 385-1215) and 811 ng/L (range: 574-1114), respectively. The decline in serum concentration of VEGF between wk 0 and 4 was significant (P < 0.05) but the over time change was not significant.

DISCUSSION

The immediate decrease in frequency of bowel movements and the improvement of the general well-being of these patients under steroid medication were similar to previous reports in IBD, including CC[25,26]. After four

weeks of treatment with the oral steroid prednisolone, the concentrations of ECP, bFGF, and VEGF in rectal perfusion fluids were reduced significantly. There were correlations between ECP, bFGF, VEGF and albumin concentrations initially, but these correlations disappeared at wk 4.

Local infiltration of the colonic mucosa by inflammatory cells is one of the histopathological characteristics of CC, and its composition includes eosinophils. The activation of eosinophils is supported by perfusion studies on UC and CC patients, where significantly increased ECP concentrations were found in these patients compared to controls[23,27,28]. Other studies have confirmed that eosinophils are activated and participate in the inflammatory process of CC[29,30]. The pro-inflammatory and profibrotic actions of eosinophils can be down-regulated by different medications including glucocorticoids[19-21]. In the present study, six, one and none out of 11 patients had ECP concentrations above the detections level at wk 0, 1 and 4, respectively. This decrease of ECP perfusate concentrations, according to Wilcoxon signed rank test between wk 1 and 4, suggests that glucocorticoids significantly reduce the secretion of ECP in the colonic mucosa of CC patients. The absence of significant difference when the over time changes in ECP concentrations according to Friedman's ANOVA did not contradict the finding because ECP median values were equal at wk 1 and 4. This happened because all the perfusate ECP values at wk 1 and 4 were below the detection level, with the exception of one case at wk 1. The anti-inflammatory actions of corticosteroids are numerous, including enhancement of eosinophilic apoptosis, increase of tissue eosinophilic clearance, deactivation of eosinophils and diminished recruitment of eosinophils as mentioned above[19-21]. The reduced perfusate concentrations of ECP found in this study, suggests that corticosteroids diminish the mucosal availability of ECP in CC. Corticosteroid treatment reduces the number of eosinophils in the colonic mucosa of CC, as shown in a small previous study[31]. Furthermore, our observations in CC resemble airway eosinophilia in asthma, which is quickly reduced by steroid treatment[32,33]. In a similarly designed therapeutic intervention study on ulcerative proctitis, the local release of ECP, EPO and MPO dropped significantly under steroid medication[34]. Contrary to CC, the numbers of neutrophils in the inflammatory infiltrate of UC is very high and this is well reflected by the high MPO concentrations found in the perfusion fluids of UC patients[35,36]. The similarity in design and the difference in results between these studies suggest that the role of neutrophils in the pathogenesis of CC is probably of little importance, except in cases where acute exacerbation of the disease cannot be excluded[37].

The bFGF concentrations in the rectal perfusates decreased 3 times from wk 0 to 1 and 11 times from wk 0 to 4. The significant downfall of intraluminal bFGF concentrations and the disappearance of the correlation between bFGF and ECP, imply that steroids markedly decrease mucosal bFGF secretion. Based on our previous findings[16] that local bFGF secretion is increased in CC patients, we suggested that bFGF enhances collagen synthesis in CC by stimulating pericryptal myofibroblasts. Furthermore, *in vitro* experiments showed that bFGF stimulated fibroblasts/myofibroblasts, collected from IBD patients, to produce more collagen, compared to corresponding cells from controls[12]. These observations and the findings in the present study suggest pericryptal myofibroblasts are synthetically active in CC[38,39], are de-activated by corticosteroids and this may be the reason behind the diminished collagen layer observed in corticosteroid-treated CC[30]and asthma patients[31]. The presence of correlations between bFGF and ECP perfusate concentrations in this study, suggests that bFGF, separately or in relation with other proteins, perpetuates chronic inflammation and triggers tissue remodeling in CC.

VEGF concentrations in the perfusion fluid were halved at wk 4 after the start of steroid treatment in CC patients. The amount of VEGF concentrations decreased significantly, as was the case for ECP, bFGF and albumin. Correlations existed between VEGF and ECP, bFGF and albumin. The correlation between VEGF and ECP disappeared at the end of this study, indicating a down-regulating effect of corticosteroids on eosinophils. VEGF and ECP are produced by eosinophils[7,29] and less activated eosinophils entail reduction of released cytokines. There is evidence that the synergetic relationship between VEGF and bFGF depends on the bFGF property to upregulate the expression of VEGF receptors (flt-1 and KDR)[40]. The correlations found between these mediators in this study may have reflected this synergism. The reduction of VEGF secretion may not only depend on steroids and de-activation of eosinophils, but also on deactivation of other VEGF producing cells, such as fibroblasts and other mesenchymal cells. This is in line with previous studies[24], which concluded that VEGF participates not only in the inflammatory process of UC and Crohn´s disease[15,41,42], but also in CC.

The clinical improvement during corticosteroid medication occurred simultaneously with a reduction in mediator concentrations, but no statistically significant correlation was found in relation to the decline in frequency of bowel movements (data not shown). In the present study, the patients themselves were the controls because the first perfusion (control) took place before starting steroid treatment.

The luminal release of albumin decreased, as in the case of ECP, significantly from wk 0 to 4 while the corresponding changes over time analyzed by the Friedman's ANOVA, did not show any significant difference. This may be due to the small number of patients included in this study. This finding, together with the correlation of VEGF and albumin, supports the hypothesis that VEGF enhances mucosal permeability in CC as previously suggested[24]. The disappearance of this correlation at wk 4 suggests that corticosteroids normalize permeability in CC. The correlation between bFGF and albumin indicates that bFGF might play a role in the increased colonic permeability. The appearance of a negative correlation at wk 4 is most probably caused by the numbers of bFGF values under detection level which have the same value, increased between wk 0 and

4 from two to six (Figure 1). Because the bFGF values under the detection levels are in the majority, the relevance of this correlation is questionable. The VEGF and ECP serum concentrations declined after introduction of corticosteroid treatment according to the Wilcoxon signed rank test but not according to Friedman's ANOVA. This may reflect a corticosteroid effect on blood eosinophils, as we did not find any difference in previous studies between the serum concentration of VEGF and ECP in CC patients, compared with controls[23,24]. This finding once again stresses the relevance of methods such as perfusion technique in studying predominantly locally restricted mucosal inflammatory processes, especially in CC.

In summary, our study confirms that the secretion of ECP, bFGF and VEGF is increased in CC and we suggest that corticosteroids relieve symptoms by decreasing the availability, release and functions of these inflammatory mediators in colonic mucosa of CC patients.

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RAPID COMMUNICATION

Hepatitis B genotypes: Relation to clinical outcome in patients with chronic hepatitis B in Saudi Arabia

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Abstract

AIM: To identify the most common hepatitis B virus (HBV) genotype in Saudi Arabia, and correlate the prevailing genotypes with the clinical outcome of patients.

METHODS: Patients were consecutively recruited from the hepatology clinics of two tertiary care referral centers. Patients were categorized into 4 different groups: group 1, patients with hepatitis B and normal liver enzymes; group 2, patients with hepatitis B and abnormal liver enzymes but without cirrhosis; group 3, patients with hepatitis B and liver cirrhosis; group 4, patients with hepatitis B and hepatocellular carcinoma. All patients had a positive hepatitis B surface antigen (HBsAg). Genotyping of HBV was performed by nested PCR-mediated amplification of the target sequence and hybridization with sequence-specific oligonucleotides.

RESULTS: Seventy patients were enrolled in this study. They were predominantly male (72.9%) in their mid-forty's (mean age 47 years). Forty-nine (70%) patients were hepatitis B envelope antigen (HBeAg) negative. The majority of patients (64%) acquired HBV through unknown risk factors. Hepatitis B genotyping revealed that 57 patients (81.4%) were genotype D, 1 patient (1.4%) had genotype A, 1 patient (1.4%) had genotype C, and 4 patients (5.7%) had genotype E, while 7 patients (10%) had mixed genotype (4 patients ADG, 1 patient DE, 1 patient DF, and 1 patient ADFG). Based on univariate analysis of genotype D patients, significant predictors of advanced liver disease were age, gender, aspartate transaminase, alanine transaminase, albumin, bilirubin, and alkaline phosphatase (all $P < 0.001$). In multivariate analysis decreased hemoglobin ($r = -0.05$; 95% CI: -0.08 to -0.03; $P = 0.001$) and albumin levels ($r = -0.004$; 95% CI: -0.007 to -0.001; $P = 0.002$) were highly significant predictors of advanced liver disease. In patients with HBV genotype D, HBeAg negativity was found to increase across advancing stages of liver disease ($P = 0.024$).

CONCLUSION: This study highlights that the vast majority of Saudi patients with chronic hepatitis B have genotype D. No correlation could be observed between the different genotypes and epidemiological or clinical factors. The relationship between genotype D and HBeAg status in terms of disease severity needs to be further elucidated in larger longitudinal studies.

Key words: Hepatitis B virus; Genotype D; Cirrhosis; Hepatocellular carcinoma; Saudi Arabia

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INTRODUCTION

Hepatitis B virus (HBV) infection is a global health problem with over 350 million chronic carriers of the virus with the risk of developing chronic hepatitis, cirrhosis or hepatocellular carcinoma (HCC). HBV is a circular, partially double-stranded DNA virus of approximately 3200 nucleotides. This highly compact genome contains four open reading frames encoding the envelope (PreS1, PreS2, S), core (core, precore), polymerase, and X proteins[1]. HBV genotypes represent naturally occurring strains of HBV that have evolved over the years and reflect the geographical distribution of HBV throughout the world. Up to now, eight different HBV genotypes have been identified and shown to cluster in different areas of the world[2,3]. They display an 8% inter-group divergence in the complete nucleotide sequence of HBV and differences

in the nucleotide homology of the surface gene, which result in different hepatitis B surface antigen (HBsAg) serotypes[4-6].

Genotype A is mainly found in Northwestern Europe, North America and Africa[7], whereas genotypes B and C have been described in South-Eastern Asian populations[8,9]. Genotype E and F are seen in East Africa and the New World, respectively. Genotype D is most often found in southern Europe, parts of Central Asia, India, Africa and the Middle East. Genotype G is a recently determined genotype in France, America, and Germany while genotype H has been reported in patients from Central America[10,11].

Recently, a number of publications have examined the impact of HBV genotype on disease pathogenesis and the clinical outcomes in patients with chronic hepatitis B. Most of these natural history studies, in view of the bimodal distribution of HBV genotypes in Asia and Western countries, have compared either genotypes B and C[11,12], or genotypes A and D[13]. The clinical impact of HBV genotypes when studied in Indian patients with a mixed population of both genotypes A and D, has shown that genotype D has a higher likelihood of developing advanced cirrhosis compared to genotype A[13].

Chronic hepatitis B is an important medical problem in Saudi Arabia although, with the implementation of HBV vaccination of children, the prevalence has dramatically reduced from 6.7% in 1989 to 0.3% in 1997[14,15]. However, little is known about the prevalence and distribution of HBV genotypes in Saudi Arabia. Furthermore, the association between the distinct genotypes and the severity of liver disease in the country remains unreported. The epidemiological studies of HBV genotypes arising from the Middle-Eastern region suggest that genotype D is perhaps the most common[16-20]. Accordingly, the objectives of our study were to identify the most common HBV genotype in Saudi Arabia, and to elucidate the relationship between the prevailing genotypes with the clinical outcome of patients.

MATERIALS AND METHODS

Enrollment of study cohort

Patients were recruited prospectively from two tertiary-care hepatology clinics at King Khalid University Hospital and the Riyadh Military Hospital in Riyadh, Saudi Arabia. These medical centers serve as referral centers for population groups resident in different geographical regions of the country. The Medical Ethics Committee in both centers approved the study protocol and all patients signed an informed medical consent indicating agreement to participate in this study. Results of all abnormal tests were provided to patients or their immediate relatives, as was deemed appropriate.

Between May 2004 to December 2005, 70 consecutive adult HBsAg positive patients were recruited at the two centers. Patients were interviewed in person by participating investigators (AAA, FMS, NA, KS) at recruitment by using a structured questionnaire. Information on socio-demographic characteristics, alcohol consumption, personal medical and surgical history, time of disease diagnosis, area of birth and upbringing, and family history of liver disease or cancers was collected. Patients who were HBsAg positive for a period exceeding 6 mo and who had not received any antiviral therapy for HBV in the preceding 6 mo were included in the study.

Serological evaluation

HBV markers [HBsAg and antibody to HBsAg (HBsAb)] were measured using standard commercial assays. In addition, further serological testing for hepatitis B envelope antigen (HBeAg) and anti-HBe antibody was also performed using the same commercial kits. HBV DNA was determined by a sensitive PCR based assay (COBAS Amplicor; Roche Diagnostics) with a lower limit of detection of approximately 200 copies/mL.

Virologic testing

Genotypic testing was performed in only those with a detectable HBV DNA (qualitative) in serum. HBV genotyping was determined from serum samples by performing nested PCR-mediated amplification of the target sequence and hybridization with sequence-specific oligonucleotides at Bioscientia Laboratory in Germany.

Study design

The patients were recruited into four groups: group 1, patients with hepatitis B and normal liver enzymes; group 2, patients with hepatitis B and abnormal liver enzymes and no laboratory or radiological features of cirrhosis; group 3, patients with liver cirrhosis secondary to hepatitis B; and group 4, patients with hepatitis B and HCC. General exclusion criteria included: (1) anti-HCV antibody positive; (2) identifiable other causes of chronic liver disease defined as (high serum iron and ferritin, abnormal serum ceruloplasmin, history of significant alcohol consumption, antinuclear antibody > 1:320, antismooth muscle antibody > 1:320, antimitochondrial antibody > 1:40); (3) history of hepatotoxic medications in the preceding three months of presentation; (4) history of antiviral therapy in the last 6 mo.

Group specific inclusion criteria for group 1 (HBV with normal liver enzymes) were: (1) persistently normal alanine transaminase (ALT) and aspartate transaminase (AST) (normal ALT and AST on at least two occasions separated by at least 3 mo), (2) normal serum bilirubin, albumin and International Normalized Ratio (INR), (3) normal complete blood count (CBC), (4) normal abdominal ultrasound (US) without features of liver cirrhosis or portal hypertension. Inclusion criteria for group 2 (HBV with abnormal liver enzymes) included: (1) persistently elevated ALT and AST (more than two times the upper limit of normal on at least two occasions separated by at least 3 mo), (2) normal CBC, (3) normal US without features of cirrhosis or portal hypertension. Patients were excluded from groups A and B if there was any abnormality in the CBC or any signs of cirrhosis or portal hypertension on abdominal US. Inclusion criteria for group 3 (HBV with liver cirrhosis) included: Any four of the following features of cirrhosis (1) platelet count < 100×10^9/L, (2) evidence of esophageal varices on endoscopy, (3) ultrasonographic features consistent with cirrhosis, (4)

albumin level less than 30 g/L, (5) INR more than 1.4 and (6) bilirubin level more than 30 μmol/L. Patients were also included in this group if there was histological evidence of liver cirrhosis regardless of the above criteria. Inclusion criteria in group 4 (HBV with HCC) included: Evidence of HCC defined as two of the followings: (1) α-fetoprotein > 400 ng/L, (2) liver mass detected by triphasic computed tomography (CT) or magnetic resonance imaging (MRI) of the abdomen, (3) fine needle aspiration (FNA) or liver biopsy showing HCC.

Ascertainment of cirrhosis and hepatocellular carcinoma

All participants had screening abdominal ultrasonography (US) at the time of recruitment into the study. The US was performed and interpreted by trained radiographers according to a standardized protocol, and the records reviewed by the investigators. Cirrhosis was diagnosed ultrasonographically based on the appearance of the liver surface, liver parenchymal texture, portal vein size, splenic size, presence of ascites and varicose veins in the portal and perisplenic area.

All patients with an α-fetoprotein > 400 ng/L (or a persistently rising α-fetoprotein) or with high clinical suspicion of HCC underwent a CT and/or MRI of the liver. The diagnosis of HCC was based upon the two published European guidelines towards the diagnosis and management of HCC[21,22]. Enhancement of a liver lesion during the arterial phase and contrast washout during the portal phase, in patients with background cirrhosis secondary to HBV was considered diagnostic of HCC. FNA or liver biopsy was obtained only where considerable doubt existed towards HCC diagnosis.

Statistical analysis

Descriptive statistics are summarized as mean ± SD. For continuous variables, ANOVA was used for comparison of the four groups. Fisher's exact or chi-square test was used for categorical variables. Univariate analysis was performed to identify important baseline characteristics associated with genotype D. Variables examined included age, gender, White blood cells, hemoglobin, platelet count, HBeAg status, albumin, ALT, AST and bilirubin. A multivariate logistic regression model was developed using forward model to assess the effect of baseline variables on disease advancement. All tests were two sided with a 5% level of significance. All analyses were performed using STATS 9.1.

RESULTS

Seventy patients were enrolled in the period from May 2004 to Dec 2005. Mean age of the patients was 47 years, and 51 patients (72.9%) were male while 19 (27.1%) were female. All geographical regions of the Kingdom were represented, with 51.5% from the central region where the vast majority of the Saudi population reside and also where the study was conducted; 21.4% from the southern region, 8.5% from the eastern region, 13% from the western region and 5.6% from the northern region, vastly representing the population distribution across the country. There were 17 patients included in group 1, 22 patients in group 2, 19 patients in group 3, and 12 patients in group 4. The majority (45 of 70 patients, 64%) had acquired HBV through unknown risk factors, while 11 patients (15.7%) reported blood transfusion, 7 (10%) reported a prior history of surgery or dental procedures and 4 (5.7%) reported a family history of HBV infection.

Table 1 Biochemical, hematological and virological parameters of all enrolled patients distributed across the four groups

Parameter (mean value)	Group 1 (*n* = 17)	Group 2 (*n* = 22)	Group 3 (*n* = 19)	Group 4 (*n* = 12)	*P*
ALT (U/L)	41.88	177.7	52	96	0.001
AST (U/L)	22	80	55.3	150	0.001
ALP (U/L)	100	111	116.4	238.8	0.001
INR	1.3	1.1	1.4	1.2	0.003
Bilirubin (μmol/L)	10	13.8	40.4	30	0.001
Albumin (g/L)	40	37.6	28.9	25.4	0.005
WBC (10^9/L)	7.1	6.1	4.9	7.2	0.160
Hemoglobin (g/L)	125	129.6	118.4	62	0.740
Platelets (10^9/L)	253	202	121.3	213	0.230
HBV DNA level					
< 200 copies/mL	5	2	3	1	NS
200-10^5 copies/mL	10	8	8	8	NS
> 10^5 copies/mL	2	12	8	3	NS

ALT: Alanine transaminase; AST: Aspartate transaminase; ALP: Alkaline phosphatase; GGT: Gamma glutamyl transpeptidase; WBC: White blood cells.

Most patients (52 of 70 patients, 74%) did not express HBeAg including 65% (11 of 17 patients) in group 1, 55% (12 of 22 patients) in group 2, 95% (18 of 19 patients) in group 3, and 92% (11 of 12 patients) in group 4. Amongst the patients who were HBeAg positive, only two patients (11.1%) had cirrhosis or HCC. The vast majority of HBeAg positive patients were either carriers with normal ALT (6 of 18 patients, 33.3%), or raised ALT (10 of 18 patients, 55.6%). One patient expressed HBeAg as well as anti-HBe. Two patients were negative for both markers. In group 1, 15 patients (88.2%) had HBV DNA < 10^5 copies/mL and were also HBeAg negative. Two patients (11.8%) had HBV DNA > 10^5 copies/mL and were also HBeAg positive. In group 2, 9 patients (41%) had an HBV DNA < 10^5 copies/mL and were all HBeAg negative. In group 3, 11 patients (58%) had an HBV DNA of < 10^5 copies/mL and all were also HBeAg negative. In group 4, 9 patients (75%) had an HBV DNA of < 10^5 copies/mL and all were also HBeAg negative. Although the proportion of patients with a high viral load (> 10^5 copies/mL) was greater in groups 2 and 3, this did not reach statistical significance (Table 1).

Table 2 shows the genotyping results according to the clinical status of the studied patients. The majority of the patients were genotype D (57 of 70 patients; 81.4%), with 58.8% (10 of 17 patients) in group 1, 95.5% (21 of 22 patients) in group 2, 84.2% (16 of 19 patients) in group 3, and 83.3% (10 of 12 patients) in group 4. One patient (1.4%) had genotype A, and one (1.4%) had genotype C. Four patients (5.7%) were genotype E, and 7 patients (10%) were mixed genotypes (4 patients ADG, 1 patient DE, 1 patient DF, 1 patient ADFG). There were no significant differences between groups in terms of

Table 2 HBV genotype distribution across different stages of liver disease and in relation to HBeAg status in 70 patients

Study cohort (*n*)	HBV genotype % (*n*)				
	D	E	A	C	Mixed
Group 1 (17)	58.8 (10)	-	5.9 (1)	-	35.3 (6)
Group 2 (22)	95.5 (21)	-	-	4.5 (1)	-
Group 3 (19)	84.2 (16)	15.8 (3)	-	-	-
Group 4 (12)	83.3 (10)	8.3 (1)	-	-	8.3 (1)
HBeAg + (18)	88.9 (16)	-	5.6 (1)	5.6 (1)	-
HBeAg - (52)	78.8 (41)	7.7 (4)	-	-	13.5 (7)

genotypes. In addition, we found no difference between different genotypes in terms of patient age, gender, area of upbringing, liver enzyme level, or serum albumin level. HBeAg negative genotype D patients comprised 50%, 57%, 93.8% and 75% across groups 1, 2, 3, and 4 respectively ($P = 0.024$) (Table 3).

The baseline characteristics according to disease severity (groups 1 to 4) are listed in Table 1. Patients in group 4 (HCC) were more likely to have a higher level of alkaline phosphatase (ALP) than those in groups 1, 2 and 3 respectively, (238 *vs* 100, $P = 0.004$; 95% CI 45-230), (238 *vs* 111, $P = 0.003$; 95% CI 45-208), (238 *vs* 116, $P = 0.036$; 95% CI 6.8-201), and also a higher level of AST than those in groups 1, 2 and 3 respectively (150 *vs* 22, $P = 0.001$; 95% CI 52-203), (150 *vs* 80, $P = 0.06$; 95% CI 3-145), (150 *vs* 60, $P = 0.02$; 95% CI 13-65). Furthermore, group 4 (HCC) patients were more likely to have a significantly lower level of albumin. In addition, 83.3% (10 of 12) of the patients in group 4 were genotype D compared to 81.4% (57 of 70) of the recruited patients who expressed the same genotype ($P = 0.30$). However, there was no significant difference in WBC, hemoglobin, and platelet count between the four groups.

Table 3 depicts the patients with genotype D according to disease severity. Patients in group 4 were older (mean age 61.5 years), tended to have higher levels of ALT, AST, and bilirubin, and lower levels of albumin. These differences were significant between the groups (all $P = 0.001$). However, there was no significant difference between the four groups with respect to hemoglobin ($P = 0.70$), platelet count ($P = 0.11$) and WBC ($P = 0.10$). In view of the small sample size in each group we did not perform a head-to-head comparison between the groups.

In univariate analysis of genotype D patients, age, gender, AST, ALT, albumin, bilirubin, and ALP were significant predictors of advanced liver disease (all $P < 0.001$). However, in multivariate analysis decreased hemoglobin ($P = 0.001$) and albumin levels ($P = 0.002$) were highly significant predictors of advanced liver disease (Table 4).

Table 3 Hepatitis B virus genotype D patients' biochemical, hematological and virological parameters distributed across the four groups (mean ± SD)

Parameter	Group 1 (*n* = 10)	Group 2 (*n* = 21)	Group 3 (*n* = 16)	Group 4 (*n* = 10)	*P*
Age (yr)	45 ± 18	35 ± 9.9	52.1 ± 7.7	61 ± 12	0.015
ALT (U/L)	40.3 ± 8	176.9 ± 100.7	54.1 ± 30.4	99.3 ± 79.9	0.001
AST (U/L)	21.1 ± 6.5	78.6 ± 56.8	63.8 ± 48.5	158.3 ± 167.6	0.001
ALP (U/L)	98.6 ± 39.9	112.6 ± 41	143.8 ± 6.2	239.9 ± 201	0.001
Bilirubin (μmol/L)	10.6 ± 4.9	14.1 ± 10.2	48.6 ± 60.4	27.8 ± 23.2	0.001
Albumin (g/L)	41.5 ± 3.2	37.8 ± 4.6	27.8 ± 7.9	24.7 ± 8.4	0.007
WBC (10^9/L)	7.5 ± 2.2	6.3 ± 1.7	6.12 ± 3.1	6.2 ± 1.9	0.100
Hemoglobin (g/L)	127.7 ± 42.5	129.2 ± 57.5	65.5 ± 55.9	62.5 ± 54.5	0.704
Platelets (10^9/ L)	250 ± 84.3	207.2 ± 56	130.8 ± 56	217.6 ± 97.4	0.113
HBeAg -/+	5/5	12/9	15/1	9/1	0.024

ALT: Alanine transaminase; AST: Aspartate transaminase; ALP: Alkaline phosphatase; GGT: Gamma glutamyl transpeptidase; WBC: White blood cells.

Table 4 Predisposing factors for advanced hepatitis B virus genotype D liver disease

	Variable	Coefficient	95% CI	*P*
Univariate analysis	Age	-0.01	-0.02 to 0.0005	< 0.001
	Gender	0.5	0.03 to 1.1	< 0.001
	AST	0.2	0.09 to 0.3	< 0.001
	ALT	0.04	0.01 to 0.03	< 0.001
	ALK	0.01	-0.001 to 0.02	< 0.001
	Albumin	-0.2	-0.3 to -0.9	< 0.001
	Bilirubin	0.08	0.0009 to 0.1	< 0.001
Multivariate analysis	Hemoglobin	-0.05	-0.08 to -0.03	0.001
	Albumin	-0.004	-0.007 to -0.001	0.002

CI: Confidence interval; ALT: Alanine transaminase; AST: Aspartate transaminase; ALP: Alkaline phosphatase.

DISCUSSION

Chronic hepatitis B is an important medical problem in Saudi Arabia. Al-Faleh *et al* in the late 1980's showed that up to 7% of Saudi children were positive for HBsAg[14]. After the introduction of universal vaccination of all Saudi children in 1989, the incidence of hepatitis B infection has declined to as low as 0.3%[15]. In spite of this dramatic decline, the burden of decompensated liver disease secondary to hepatitis B is expected to increase significantly in the next 40 years as the previously infected children start aging.

Hepatitis B genotyping has received immense attention recently and its clinical implications are being investigated extensively throughout the world. This study is the first to show that genotype D is the most prevalent genotype in Saudi Arabia. In this prospective study all regions of the country are represented as well as the entire spectrum of chronic HBV infection ranging from the carrier state to HCC. Our study concurs with reports from other parts of the Middle East like Egypt, Yemen, Turkey, Iran, and Tunisia[16-20], all showing that genotype D is the most common genotype in this region.

HBV genotypes may contribute in part to the wide variation in prevalence rates of HBV infection in different parts of the world through difference in rates of replication and ability to evade immune clearance. However, studies comparing the replication capacity and immune response of the various HBV genotypes have not been performed. Nevertheless, many studies have shown a strong relationship between HBV genotypes and mutations in the precore and core promoter regions that abolish or

diminish the production of HBeAg[23-26]. In our population which is predominantly HBeAg negative (presumably indicating pre-core or core promoter mutation), the majority of patients had genotype D thereby supporting the above observation.

Hepatitis B genotypes have been correlated with various epidemiological, virological, and clinical variables. It has been recently reported from China that patients with genotype B have a lower prevalence of HBeAg than those with genotype C[12]. Several other studies reported a correlation between HBV genotype and HBeAg clearance. Similarly in our study, we found a lower prevalence of HBeAg among our patients with genotype D (28%), suggesting that HBeAg clearance occurred at higher rates among patients with genotype D.

Correlation between the clinical outcomes of patients with HBV and their genotypes has also been reported. One study found that HBsAg carriers with genotype B had lower histological activity scores[23]. Three other studies involving a total of 939 Chinese patients with chronic HBV infection found that genotype C was more prevalent in patients with cirrhosis[27-29]. In our study, while 58.8% in group 1 (HBV with normal ALT) were genotype D, the number of patients with the same genotype across advancing stages of liver disease, in groups 2, 3, and 4 comprised 95.5%, 84.2%, 83.3% respectively ($P = 0.4$). These findings suggest that genotype D does not correlate with advancing liver disease. However, this could be related to the small sample size of the present study, and probably due to the predominance of genotype D in all clinical forms. Further analysis in large-scale longitudinal studies is required to better delineate this relationship.

In our patients with HBV genotype D, HBeAg negativity was found to increase significantly across more advancing stages of liver disease ($P = 0.024$). Previous studies have revealed that HBeAg positive genotype C[6] or genotype A[30] patients were more likely to have active liver disease. Progress of liver disease in relation to HBeAg status has not been reported as yet in the various studies dealing with genotype D[13,19,20]. Although this observation is significant, the association between HBeAg status and genotype D, in terms of severity of liver disease needs to be studied further before any additional conclusions can be derived.

Since predictors of advanced HBV liver disease were described before as a whole or in relation to individual genotypes other than in genotype D[31], our study describes for the first time these predictors in genotype D. Hemoglobin and albumin levels are independent predictors of advanced liver disease. Furthermore, group 4 patients (HCC) with genotype D showed significantly higher biochemical parameters (AST, ALT, and bilirubin) and lower albumin levels compared to early stage liver disease.

The relation between HBV genotype and HCC is inconclusive. One study found that genotype B was associated with hepatocellular carcinoma at an earlier age[28], but this finding was not confirmed by other studies[11,27,32]. Another study in Indian patients reported that genotype D was commonly found to be associated with HCC in patients < 40 years of age[13]. However, in our study, none of our HCC patients with genotype D (10 patients) were < 40 years of age (mean age 61 ± 12).

In Saudi Arabia HCC is the second most common cancer in men[33], and in a country where hepatitis B is endemic this could imply a correlation between the most prevalent genotype (D) and HCC. In our patients, 83.3% of the patients in group 4 (HCC) were genotype D compared to 81.4% of the overall number of recruited patients who expressed the same genotype. This figure did not reach statistical significance ($P = 0.30$). Moreover, since HBV is thought to be directly carcinogenic because of the integration of HBV DNA into the cellular DNA of the host[34], it may also explain the observed lack of correlation between genotype D and the development of HCC.

There were no significant differences between groups in terms of genotypes. This is likely secondary to the fact that the vast majority of patients had genotype D making comparisons with the other relatively rarer genotypes difficult. Along with the limitations imposed by the small sample size across the different groups, the study was also restricted by the absence of histology in groups 1 and 2, thereby possibly misallocating some of these patients into either more or less active groups.

In conclusion, genotype D is the most common genotype in Saudi Arabia. Because of the fact that the vast majority of the patients have genotype D, no correlation could be observed between different genotypes and epidemiological or clinical factors. A large-scale study is required to obtain further information on the role of genotype D and its impact on the progress of liver disease.

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RAPID COMMUNICATION

Hepatitis C virus transmission and its risk factors within families of patients infected with hepatitis C virus in southern Iran: Khuzestan

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Abstract

AIM: To determine whether hepatitis C virus (HCV) infection of index cases increases intrafamilial transmission (sexual and nonsexual contacts) of HCV.

METHODS: In a case-control descriptive study we enrolled 300-household contacts of 60 index cases (40 males and 20 females) of HCV infection and 360 pair-matched controls in Ahwaz JundiShapour University Hospitals from August 1, 1998 to September 1, 2003. The control group consisted of first time blood donors referred to the Regional Blood Transfusion Organization. Serum samples and demographic data and a medical history including the existence of risk factors for HCV (after a questionnaire on the risk factors for parenteral exposure) were obtained from each subject. Antibodies to HCV were detected employing a commercially available second-generation enzyme immunoassay (EIA, Abbott Ⅱ). Positive serum specimens were retested using a second-generation recombinant immunoblot assay (RIBA-2) and a polymerase chain reaction for HCV RNA. Data analysis was carried out for intra-household clustering.

RESULTS: Only 4 of 300 (1.33%) cases of household contacts without percutaneous risk factors were positive for HCV Ab while the remaining 296 family contacts were negative for anti-HCV. The mean age of the index cases was 28.4 (Std 15.22) years. The anti-HCV prevalences in parents, spouses, children of the index cases were 0.87% (1/115), 3.39% (2/59)) and 0.79% (1/126), respectively. Among couple partners negative for anti-HCV antibodies, the mean duration of the sexual relationship was 6 years. The two-couple partners positive for anti-HCV antibodies married the index cases for longer than 15 years. The prevalence of positive HCV Ab among household contacts (1.33%) was not significantly higher than that in the controls (1%) ($P > 0.06$).

CONCLUSION: Intrafamilial transmission of HCV is not the significant transmission route and sexual transmission does not seem to play a role in the intrafamilial spread of HCV infection. Intrafamilial transmission of HCV is possible but occurs at a low rate.

Key words: Intrafamilial transmission; Hepatitis C virus; Khuzestan; South-west of Iran

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INTRODUCTION

One of the most substantial problems in public health is hepatitis C virus (HCV) infection, which affects approximately 1%-5% of the world's population and occurs in all countries. Epidemiological information on HCV is essential for strategic prevention of chronic hepatitis, liver cirrhosis and cancer. The rate of HCV infection differs in particular countries. The prevalence in developed countries amounts to 0.2%-2.2%, while in developing countries it reaches 7%. In some regions or in risk groups the rate of occurrence may be as high as 30%-90%[1,4].

HCV infection is acquired mainly parenterally by transfusion of infected blood, rupture of the continuity of skin or mucous membrane, infected medical equipment despite strict hygienic control, intravenous drug abuse, hemodialysis or organ transplantation. HCV infection is an important cause of post-transfusion hepatitis. Transmission through sexual contacts has been implicated, although this may be a rather inefficient mode of transmission[2,3]. HCV has also been detected in persons in whom no clear risk factor has been defined, and these cases constitute about 40%-45% of HCV infections[4]. According to

Table 1 Demographic and clinical information on HCV-infected household members

Household members	*n*	Age (yr)/sex	Medical history	Clinical liver disease	Transfusion	HCV RNA
Nonsexual household contact (Mother)	1	58/F	None	No	No	Negative
Sexual partners of index case	1	43/F	None	No	No	Positive
Wife	1	38/F	None	No		Negative
Nonsexual household contact (Daughter)	1	23/F	None	No	No	Positive

the published data, the prevalence of HCV infection in Iran is 0.59%-0.8%[5]. Mother-to-infant transmission has also been demonstrated[6] but the possibility of other transmission routes has not been thoroughly explored. With the use of RT-PCR or bDNA techniques, HCVRNA has been detected in many systemic fluids other than in blood, including peritoneal effusion, seminal and vaginal secretion, urine, feces and typhoid secretion. At least 20% of hepatitis C patients develop cirrhosis with the associated risk of developing hepatocellular carcinoma (HCC)[7]. Despite primary hepatotropism, HCV can affect tissues and organs such as kidneys, thyroid, salivary glands, eyes, or the hematopoietic and lymphatic systems other than the liver[8]. HCV infection seems to be connected with several autoimmune diseases[9].

To our knowledge little information is available about HCV infection due to contacts of patients infected with HCV in southern Iran: Khuzestan. Because HCV may be transmitted by the non-parenteral routes such as sexual and non-sexual household contacts, this study was undertaken to investigate whether intrafamilial transmission occurs *via* the usual contacts between patients and their household members who are unaware of the potential infectious state of the patients, to determine the prevalence of antibody to HCV in the contacts of HCV positive cases (index patients) and to evaluate the potential risk factors associated with intrafamilial transmission of HCV.

MATERIALS AND METHODS

Subjects

During a 5-year period in a case-control descriptive study we enrolled 300 household contacts of 60 index cases (40 males and 20 females) of HCV infection and 360 pair-matched controls in Ahwaz JundiShapour University Hospitals from August 1, 1998 to September 1, 2003. The control group consisted of first time blood donors referred to the Regional Blood Transfusion Organization.

Methods

Serum samples and demographic data and a medical history including the existence of risk factors for HCV (after a questionnaire on the risk factors for parenteral exposure) were obtained from each subject. The questionnaire consisted of questions regarding demographic variables, household behaviors and extra-familial factors including various potential parenteral exposures to blood or blood products (such as past hospital admission, operation, injuries needing hospital interventions, blood or blood product transfusion), history of parenteral injections and intravenous drips, travel history outside Iran, as well as dental treatment, tattooing and ear piercing. All these factors are known to be associated with HCV infection. All index cases and household contacts and controls answered the questionnaire and had their blood tested for anti-HCV. Antibodies to HCV were detected employing a commercially available second-generation enzyme immunoassay (EIA, Abbott Ⅱ). PCR for HCVRNA and a second-generation recombinant immunoblot assay (RIBA-2) were performed in all index cases and positive serum specimens were obtained from each subject. The Amplicor HCV RNA assay was used to detect HCV RNA in index case serum (Roche Diagnostic Systems). None of our cases (household contacts and index cases) revealed any signs of HBV infection (MEIA, Abbott IMX) or any other causes of acute or chronic liver diseases such as HAV, EBV and CMV infections, autoimmune diseases, alcohol and drug abuse, α1-antitrypsine deficiency, Wilson' s disease, or hemochromatosis. All these index cases were still receiving follow-up at the time of our study.

Statistical analysis

The data were analyzed statistically using SPSS, version 9. Comparison was made using the Student's *t* test and chi-square test. $P < 0.05$ was considered statistically significant.

RESULTS

All the 60 index patients gave positive reactions in the second generation anti-HCV EIA. We assumed that most of them were chronically infected with HCV. A total of 300 household contacts of the index patients were subjected to the second generation anti-HCV EIA. Only 4 of 300 (1.33%) cases of household contacts without percutaneous risk factors were positive for HCV Ab while the remaining 296 family contacts were negative for anti-HCV. The mean age of the index cases was 28.4 (Std 15.22) years. The anti-HCV prevalences in parents, spouses, children of the index cases were 0.87% (1/115), 3.39% (2/59)), and 0.79% (1/126), respectively. Among the couple partners negative for anti-HCV antibodies, the mean duration of the sexual relationship was 6 years. The two-couple partners positive for anti-HCV antibodies married the index cases for longer than 15 years. PCR for HCVRNA and second-generation recombinant immunoblot assay (RIBA-2) were performed in household members of two cases, one wife and one daughter (Table 1). The prevalence of positive HCV Ab among household contacts (1.33%) was not significantly higher than that in the controls (1%) ($P > 0.06$).

DISCUSSION

HCV infection, a world-wide spread liver disease, is most

often asymptomatic in adults. It leads to serious clinical consequences that often occur later. About 50%-70% of mild infections progress to chronic phase and long term observations of natural history of the disease have confirmed that HCV is the risk factor for cirrhosis and primary hepatic carcinoma. However, there is a paucity of data on risk behaviors associated with HCV transmission through household contacts. HCV may be transmitted by the parenteral and other routes similar to that of HIV and HBV. Studies on family members of patients with chronic B hepatitis indicate that this virus can be transmitted both by vertical from mothers positive for hepatitis B surface antigen (HBsAg) and hepatitis B e antigen (HBeAg) and by horizontal (sexual and non-sexual) routes. Spouses and siblings of HBsAg-positive subjects are frequently found to have a high prevalence of serological markers of current or past HBV infection[10,11]. But the epidemiological relevance of intrafamilial transmission of HCV has not been clearly established.

This study estimated (1.33%) HCV seroprevalence among the household contacts of HCV-seropositive index cases, which is lower than the finding of other studies reporting that HCV seroprevalence is 16% and 20% among contacts of HCV-seropositive index patients[12,13]. Our results differ from those in these studies reporting a higher rate of HCV infection in the family members of adult patients with chronic hepatitis C. This difference could be attributed to one or more of the following limitations in studies, i.e. a small-sized study, inadequate duration, intensity of potential contact with adult patients, low infectivity of HCV in blood, genotypes, and unknown sensitivity of the hepatitis C radioimmunoassay used for detecting HCV infection[14]. Although intrafamilial HCV transmission through nonsexual contacts has been recognized as a major route in the Saudi population and elsewhere, the mechanisms underlying such transmissions have not been elucited. Another study has reported an elevated prevalence of 5.7% among household contacts, compared with HCV seroprevalence of 0.5% in the general population[15-17]. There are conflicting data in the literature concerning the role of sexual contacts in the spread of HCV infection. The prevalence of positive anti-HCV in spouses is different and may be a consequence of many factors such as sexual behavior and duration of marriage[18,19].

In this study, we found that spouses of anti-HCV positive patients were more likely to be infected with HCV than other family members and the infection rate increased with duration of marriage. In agreement with other authors[20], we found that positive anti-HCV increased in spouses with their length of marriage. In particular, a significant difference was found in the prevalence of positive anti-HCV between spouses married for more than 15 years and those married for a shorter time. Homology analysis on HCV nucleotide sequence is important in the study of sexual transmission of HCV[21], but we did not perform homology analysis in our studied cases. It is not easy to explain the increased rate of HCV infection in couples married for a longer time. Other authors reported that there is no prevalence in two groups of sexual partners married respectively for 13 and 15 years[3,22]. Marriage usually includes a sexual relationship, but also other kinds of body contact and exposure to the same risk factors (i.e. sharing the same personal tools such as toothbrushes, razors, dental appliances, *etc.*) as suggested by recent findings in Taiwan[20,23]. We did not perform genotyping in our studied cases but in Iran, genotype (1a) has been identified in the majority of chronic HCV patients[24]. HCV genotyping between the index cases and infected family contacts can clarify whether the infection is acquired in or out of the family setting. The HCV seroprevalence among the contacts of HCV-seropositive index patients is lower in our study than in populations elsewhere.

Our data suggest that sexual and nonsexual contacts with HCV-infected hemophilia patients within households are not associated with an increased risk for HCV infection. Further study is needed to assess the extent and the causes of nonparenteral transmission of HCV. Implementation of an education program based on the identified risk factors may reduce the spread of HCV in our area.

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RAPID COMMUNICATION

Evaluation of the role of *H pylori* infection in pathogenesis of gastric cancer by immunoblot assay

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Abstract

AIM: To elucidate the different serological reactions to *H pylori* using the immunoblotting technique for further understanding of its pathogenic role in gastric cancer.

METHODS: A total of 54 patients were divided into two groups after upper gastrointestinal endoscopy: normal control group (25 patients) and gastric cancer group (29 patients). Both groups were further divided into *H pylori* (+) and *H pylori* (-) subgroups based on the results of CLO test, Giemsa staining and culture. Sera were further analyzed with the immunoblotting technique (HelicoBlot 2.0, Genelabs Diagnostics, Singapore).

RESULTS: The positive rate of the immunoblotting test was as high as 88.9% in the *H pylori* (-) gastric cancer group and only 14.3% in the *H pylori* (-) normal control group with a statistically significant difference.

CONCLUSION: The prevalence of *H pylori* infection is higher in gastric cancer patients than in the normal controls, suggesting that *H pylori* may play a role in the pathogenesis of gastric cancer.

Key words: Western blot; Immunoblotting; Gastric cancer; *H pylori*; Enzyme-linked immunosorbent assay

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INTRODUCTION

H pylori, a Gram-negative bacterium, is now widely considered as one of the major etiologic factors in the pathogenesis of a great variety of gastrointestinal diseases such as gastritis, peptic ulcers and mucosa-associated lymphoid tissue lymphomas (MALTomas)[1]. There is increasing evidence that cancer of the stomach is also strongly associated with *H pylori* colonization[2-6]. Numerous antibodies against antigens of *H pylori* can be detected by serological analysis using the Western immunoblot technique[7-10]. Among these antibodies to *H pylori*, polypeptides with molecular masses of 116 kDa (against the cytotoxin-associated antigen, CagA), 89 kDa (against the vacuolating toxin antigen, VacA), 35 kDa, 30 kDa, 26.5 kDa and 19.5 kDa are considered as the most specific antibodies used in the diagnosis of *H pylori* infection and their corresponding antigens probably play a pathogenic role in the distinct gastrointestinal diseases. Particularly the antigens CagA and VacA not only seem to have a significant association with peptic ulcer disease but also increase the risk of developing gastric cancer[11-16]. The aim of this study was to elucidate the probable pathogenic role of *H pylori* in gastric cancer and serological stigmata of its remote infection as detected by the immunoblotting technique.

MATERIALS AND METHODS

Patients

Between March 1998 and May 2000, 54 consecutive patients (34 women, 20 men; age range: 20-70 years) who had epigastralgia and vague abdominal complaints but no remarkable past medical history of systemic diseases (such as generalized sepsis, uremia or hematologic malignancies) were recruited prospectively in this study. These patients visited the Outpatient Clinic or the Health Management Center of Shin Kong Wu Ho-Su Memorial Hospital for a routine health check-up. During upper GI endoscopy, specimens were taken from the antrum for rapid urease test, Giemsa stain and culture to elucidate the patient's *H pylori* status. When gastric malignancy was suspected, more specimens were taken from the lesion for histological examination. The patients were then divided into a normal control group (n = 25) and a gastric cancer group (n = 29) (Table 1). The normal control group and gastric cancer group were further divided into *H pylori* (+) and *H pylori* (-) subgroups. The *H pylori* (+) subgroup had positive results in at least two of the three tests, while the three tests were

Table 1 Positive rate (%) of different reaction bands in the two groups of patients

	Normal		CA		
	H pylori (+)	*H pylori* (-)	*H pylori* (+)	*H pylori* (-)	
n	11 (%)	14 (%)	11 (%)	18 (%)	*P*
Overall	100.0	14.3	100.0	88.9	< 0.0001
116 kDa	100.0	14.3	90.9	72.2	0.002
89 kDa	44.4	0.0	60.0	27.8	0.052
35 kDa	100.0	0.0	100.0	61.1	0.0003
30 kDa	100.0	7.14	81.8	58.80	0.003
26.5 kDa	90.9	14.3	72.7	66.7	0.005
19.5 kDa	72.7	0.0	40.0	16.6	0.238

Fisher's exact test was used to test for the different positive rate between CA-*H pylori* (-) and Normal-*H pylori* (-) group.

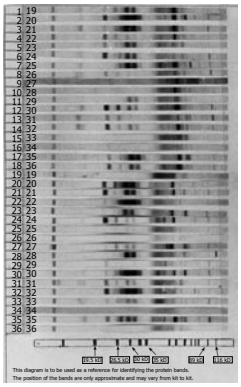

Figure 1 Example of the immunoblotting reaction bands. A reaction sheet from a group of patients enrolled in the study.

all negative in the *H pylori* (-) subgroup. In the *H pylori* (-) normal subgroup there were 12 female and 2 male patients with a mean age of 34.6 years. In the *H pylori* (+) normal subgroup there were 7 female and 4 male patients with a mean age of 37.2 years. In the *H pylori* (-) cancer subgroup there were 9 female and 9 male patients with a mean age of 58.8 years. In the *H pylori* (+) cancer subgroup there were 6 female and 5 male patients with a mean age of 59 years. In the *H pylori* (-) cancer subgroup, tumors were found in the gastric antrum, angle, corpus and cardia of 6, 3, 5 and 4 patients, respectively. Meanwhile, in the *H pylori* (+) cancer subgroup, tumors were found in the antrum of 5 patients (2 of them had tumor involving antrum and angle), in the antrum and lower corpus of 2 patients, in the angle of one patient, in the corpus of 3 patients, and in the corpus as well as fundus and cardia of one patient. Histopathological studies demonstrated that all the suspicious malignant lesions were adenocarcinoma. In order to analyze the possible link between gastric cancer and remote *H pylori* infection, the sera from patients were analyzed with the immunoblotting technique (HelicoBlot 2.0, Genelabs Diagnostics, Singapore) (Figure 1). Five reaction bands could be recognized with the immunoblot technique: 116 kDa (CagA), 89 kDa (VacA), 35 kDa, 30 kDa, 26.5 kDa and 19.5 kDa. The immunoblotting was considered as positive with the detection of one reaction band of 116 kDa (CagA) and/or 89 kDa (VacA) and/or 35 kDa (major antigens), and/or two other reaction bands (minor antigens, 30 kDa, 26.5 kDa, 19.5 kDa), as recommended by the manufacturer. In addition, sera from the *H pylori* (-) cancer group of patients were further analyzed by enzyme-linked immunosorbent assay (ELISA, Immulite *H pylori* IgG, Diagnostic Products Corporation, Los Angeles, USA), and the two serological methods were compared. The collected data were finally analyzed with the Fisher's exact test.

RESULTS

The seroprevalence of antibodies to 116 kDa (Cag-A) positive *H pylori* strain was high among the patients enrolled in this study: 100% in the normal *H pylori* (+) control group, 90.9% in the CA-*H pylori* (+) group, and also strikingly high in the CA-*H pylori* (-) group (72.2%). A quite similar finding was observed with the 35 kDa antigen (Table 1). The seroprevalence of antibodies to the third major antigen 89 kDa (VacA) was 44.4% in the normal *H pylori* (+) group, 60% in the CA-*H pylori* (+) group and 35.7% in the CA-*H pylori* (-) group. In the case of minor antigens, the seroprevalence of antibodies to the 30 kDa antigen was 100% in the normal *H pylori* (+) group, 81.8% in the CA-*H pylori* (+) group, and also remarkably high (58.8%) in the CA-*H pylori* (-) group. For the 26.5 kDa antigen, the seroprevalence of antibodies was 90.9% in the normal *H pylori* (+) group, 72.7% in the gastric cancer-*H pylori* (+) group, and 66.7% in the CA-*H pylori* (-) group. For the 19.5 kDa antigen, the seroprevalence of antibodies was 72.7% in the normal *H pylori* (+) group, and 40% in the gastric cancer-*H pylori* (+) group. When the reaction bands were equivocal (neither positive nor negative), they were considered undetermined with a prevalence of 2.2% in the 116 kDa antigen, 10.9% in the 89 kDa antigen, 2.2% in the 35 kDa antigen, 8.7% in the 30 kDa antigen, 3.6% in the 26.5 kDa antigen, and 5.8% in the 19.5 kDa antigen. These equivocal reaction bands might indicate that a low serum concentration of the corresponding antibodies was insufficient to yield a clear-cut reaction with their respective antigens. Analysis of the seroprevalence of antibodies to different antigens yielded the following overall positive rates for immunoblotting test: 100% in the normal *H pylori* (+) subgroup, 14.3% in the normal *H pylori* (-) subgroup, 100% in the gastric cancer-*H pylori* (+) subgroup and 88.9% in the gastric cancer-*H pylori* (-) subgroup, respectively. It should be pointed out that the positive rate for the immunoblotting technique was strikingly higher in the gastric cancer-*H pylori* (-) subgroup than in the normal *H pylori* (-) subgroup and there was a statistically significant difference achieved by Fisher's exact test ($P < 0.05$). This interesting finding denoted that the presence of *H pylori* as a remote infection in both *H pylori* (-) subgroups detected by immunoblotting assay was much more significant in the gastric cancer-*H pylori* (-) subgroup than in the normal *H pylori* (-) subgroup. This important issue might be overlooked if only rapid urease test, Giemsa staining and culture were performed. However, when ELISA was carried out to detect IgG to *H pylori* antigens using sera from these 18 gastric cancer-*H pylori* (-) patients, only 9 of them were positive (50% *vs* 88.9%). Since *H pylori* might not be closely implicated in the development of tumors in the cardiac region, if the four *H pylori* (-)

cancer patients with their tumor localized in the cardia were excluded from statistical analysis, the overall result was identically significant (Table 2).

DISCUSSION

The role of different *H pylori* antigens in gastrointestinal diseases still remains controversial. In contrast to Western developed countries, different reaction bands in immunoblot assay fail to predict a particular disease in Taiwanese patients[17-21]. Two *H pylori* proteins, VacA and CagA, are virulence factors which may enhance gastric mucosal damage and promote the development of peptic ulcers and gastric mucosa atrophy. By identifying different *H pylori* proteins, immunoblot assay can screen patients at high risk of developing gastrointestinal diseases, such as peptic ulcer and gastric cancer. However, the high seroprevalence of antibodies to CagA-positive *H pylori* strains in Taiwanese patients with various gastrointestinal diseases has rendered the CagA-positive phenotype, an unusable marker for screening patients with a determined disease and immunoblot assay has no predictive and diagnostic value in Taiwanese patients. ELISA may reveal a significant decrease in IgG antibody titers approximately two months after treatment with antimicrobials. In contrast, immunoblot assay may detect IgG antibodies to specific antigens such as CagA and VacA several years after treatment[22-24]. These findings suggest that ELISA is a useful quantitative tool for monitoring eradication of *H pylori* while immunoblot assay is a qualitative method able to demonstrate remote *H pylori* infections which are not detectable by ELISA. The sensitivity and specificity of ELISA may decrease with the decrease in IgG titers. The immunoblotting technique might be recommended as a confirmative test for antibodies detected by ELISA[25,26]. Furthermore, although a high accuracy has been reported in Western countries, commercial ELISA might be unsatisfactory in Asians[27]. Therefore, immunoblot assay may be regarded as a sensitive, non-invasive means for the diagnosis of *H pylori* infection. However, major serological cross-reactions with *Campylobacter jejuni* and bacterial lipopolysaccharide have been found, which might explain the false positive results, while decrease in concentration of antibodies might yield equivocal reaction bands. It is known that *H pylori* colonization causes chronic active inflammation of gastric mucosa which eventually leads to the development of atrophic gastritis, intestinal metaplasia and dysplasia. Eighty-nine percent of *H pylori* (-) patients with gastric adenocarcinoma were proven to have a *positive* immunoblot assay in this study, indicating that these patients might have been infected with *H pylori* in a certain past period of their lifetime. This interesting finding suggests that *H pylori* can be detected in hostile gastric environments such as mucosa atrophy, but its hidden remote infection is still demonstrated in serum by immunoblot assay[28]. Therefore, the role of *H pylori* in the pathogenesis of gastric cancer should be stressed. Further studies are necessary to elucidate the possible link between *H pylori* infection and mechanisms of carcinogenesis.

In conclusion, 88.9% of patients with gastric cancer in *H pylori* (-) subgroup have a positive immunoblot assay for *H pylori* infection. Immunoblot assay can disclose remote *H pylori* infection which might be overlooked if only rapid urease test, Giemsa staining and culture, or ELISA for IgG antibody, is performed.

Table 2 Positive rate (%) of the different reaction bands in the two groups of patients

	Normal		CA		
	H pylori (+)	*H pylori* (-)	*H pylori* (+)	*H pylori* (-)	
n	11 (%)	14 (%)	11 (%)	14 (%)	*P*
Overall	100.0	14.3	100.0	92.9	< 0.0001
116 kDa	100.0	14.3	90.9	71.4	0.006
89 kDa	44.4	0.0	60.0	35.7	0.04
35 kDa	100.0	0.0	100.0	57.1	0.002
30 kDa	100.0	7.14	81.8	75.1	0.01
26.5 kDa	90.9	14.3	72.7	71.4	0.006
19.5 kDa	72.7	0.0	40.0	14.3	0.482

Four *H pylori* (-) patients with their tumors localized in the cardia were excluded from the analysis. Fisher's exact test was used to test for the different positive rate between CA-*H pylori* (-) and Normal-*H pylori* (-) groups.

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RAPID COMMUNICATION

Evaluation of intraoperative radiotherapy for gastric carcinoma with D2 and D3 surgical resection

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Abstract

AIM: To study the proper sites and doses of intraoperative radiotherapy (IORT) for gastric carcinoma and the effects of this treatment.

METHODS: A total of 106 patients with stage Ⅰ-Ⅳ gastric carcinoma who received D2 or D3 radical operation combined with IORT were analyzed. Sixty-seven patients with gastric cancer of the antrum and body underwent distal gastrectomy. The sites of irradiation were at the celiac artery and hepatoduodenal ligment area. Another 39 patients with carcinoma of the cardia and upper part of the gastric body and whole stomach underwent proximal gastrectomy or total gastrectomy. The sites of irradiation for this group were the upper margin of the pancreas and the regional para-aorta. The therapeutic effects (including survival and complications) of these 106 cases received operation combined with IORT (IORT group) were compared with 441 cases treated during the same time period by a radical operation alone (operation group).

RESULTS: The radiation dose below 30 Gy was safe. The therapeutic method of the operation combined with IORT did not prolong the survival of patients with stage Ⅰ and Ⅳ gastric cancer, but the 5-year survival rates of patients with stage Ⅱ and Ⅲ gastric cancers were significantly improved. The 5-year survival rates of the stages Ⅲ cancer patients receiving D2 resection combined with IORT were markedly improved, while for those receiving D3 radical resection, only the postoperative 3- or 4-year survival rates were improved (P < 0.005-0.001). The 5-year survival rate for those patients was raised only by 4.7% (P > 0.05).

CONCLUSION: The 5-year survival rates of patients with stages Ⅱ and Ⅲ gastric carcinoma who received D2 lymphadenectomy combined with IORT were improved, and there was no influence on the postoperative complications and mortality.

INTRODUCTION

The fate of patients after surgical removal of a gastric carcinoma is determined to a large degree by regional failure of the operation (e.g. tumor recurrence in the tumor bed or in an adjacent structure). This is true for palliative resections with macroscopic tumor residues (D2) as well as for operations with microscopic involvement of resection margins (D1) or no discernible malignant cells (D0). Recurrent tumors may originate from extension of the primary tumor or from regional lymph node metastases not encompassed by the surgical procedure[1]. It is desirable therefore to develop and study additional tumoricidal measures that might eliminate residual malignant tissues, thereby increasing the chance of regional tumor control[2].

Intraoperative radiotherapy (IORT) as an adjunct to surgical excision of solid malignancies has been used, however, clear-cut evidence of its benefit for tumor control is still deficient. In IORT a large single dose of radiation is given to the area with greatest risk of local failure[3-5]. Based on the available oncologic and radiotherapeutic data, radiation thus applied should be able to control tumor growth. The biologic and logistic advantages of radiation applied directly to the tumor bed at a single dose are obvious. For these reasons any information related to the efficacy of IORT is highly desirable and welcome.

Under these considerations, we have used IORT for gastric cancer and report the clinical results in comparison with that of patients treated by operation alone. The clinical results of IORT for gastric cancer were analyzed based on the histologic findings.

MATERIALS AND METHODS

Research subjects

A total of 106 patients were treated by radical resection and IORT between 1992 and 1998, including 13 cases in

Table 1 Clinical data of gastric carcinomas in different stages classified by intraoperative radiotherapy (IORT) and operation alone

Groups	Staging	*n*	Location of tumor *n* (%)			Operation method *n* (%)					
			Antrum	Body	Cardiac	TS	D2	D3	DG	PG	TG
IORT											
	Ⅰ	13	8 (62)	2 (15)	3 (23)	0 (0)	9 (69.3)	4 (30.7)	10 (76.9)	3 (23.1)	0 (0)
	Ⅱ	17	12 (70.6)	2 (11.8)	3 (17.6)	0 (0)	6 (35.3)	11 (64.7)	13 (76.5)	3 (17.6)	1 (5.9)
	Ⅲ	48	25 (52.1)	6 (12.5)	8 (16.7)	9 (18.7)	9 (18.8)	39 (81.3)	30 (62.5)	5 (10.4)	13 (27.1)
	Ⅳ	28	13 (46.4)	2 (7.1)	5 (17.9)	8 (28.6)	3 (10.7)	25 (89.3)	14 (50)	6 (21.4)	8 (28.6)
Operation											
	Ⅰ	70	50 (71.4)	16 (22.9)	4 (5.7)	0 (0)	50 (71.4)	20 (28.6)	66 (94.3)	4 (5.7)	2 (2.8)
	Ⅱ	67	49 (73.1)	5 (7.5)	13 (19.4)	0 (0)	31 (46.3)	36 (53.7)	54 (80.6)	11 (16.4)	2 (3.0)
	Ⅲ	244	164 (67.2)	37 (15.2)	28 (11.5)	15 (6.1)	110 (45.1)	134 (54.9)	201 (82.4)	28 (11.5)	15 (6.1)
	Ⅳ	60	36 (60)	10 (16.7)	6 (10)	8 (13.3)	8 (13.3)	52 (86.7)	42 (70)	4 (6.7)	14 (23.3)

TS: Total stomach; D2: Lymph node 2 dissection; D3: Lymph node 3 dissection; DG: Distal gastrectomy; PG: Proximal gastrectomy; TG: Total gastrectomy.

stage Ⅰ, 17 in stage Ⅱ, 48 in stage Ⅲ and 28 in stage Ⅳ. There were 77 men and 29 women, aged from 31-80 years, averaging 52.4 years. Location of the carcinoma was: antrum 58, body 12, cardiac 19, and total stomach 17 cases. Operation method: D2 lymphadenectomy 27, D3 lymphadenectomy 79; distal gastrectomy 17, proximal gastrectomy 67 and total gastrectomy 22 cases. To evaluate the effectiveness of IORT, 441 patients who were treated by operation alone during the same time period were classified histologically, and their survival rates were compared with those of patients treated by IORT. In addition, a comparative study was performed on the survival rates of patients treated by IORT and those treated by operation alone based on the degree of staging (Table 1). Inclusion criteria for IORT[6] were: (a) gastric adenocarcinoma confirmed through preoperative biopsy; (b) age between 25 and 80 years; (c) World Health Organization (WHO) performance status of 0-2; and (d) the availability of IORT facilities.

Patients were excluded if they had serious concurrent illness, active infection, symptomatic ischemic heart disease, congestive heart failure, a recent history of myocardial infarction, or symptomatic arrhythmia. Patients with significant gastrointestinal disturbance that could cloud the interpretation of gastrointestinal toxicity were also excluded. In addition, patients with severe malnutrition or severe nausea, or frequent vomiting were excluded.

Surgery was performed through a median laparotomy and surgeons were allowed to use either total or subtotal gastrectomy (according to the tumor location) combined with D2 and D3 lymphadenectomy. The same two senior surgeons were in charge of the operations during the whole protocol.

Radiation location

After the gastrectomy and lymphadenectomy and before alimentary reconstruction, the local region of the hepatoduodenal ligament and the upper margin of the pancreas or the gastric bed were irradiated in 67 patients who were treated with gastrectomy in the antrum and body of the stomach. The other 39 patients with total gastrectomy underwent removal of the pancreas capsule and extended lymph node dissection including the nodes along the splenic artery. In the patients with body, cardiac and total stomach cancer, the tail and body of the pancreas were moved up to the right side margin of the abdominal aorta and the superior mesenteric vein and the region of the para-aorta received irradiation.

IORT was administered to the tumor bed and celiac axis at the time of gastric resection in those patients whose tumor appeared transmural and who were sufficiently stable to tolerate a transfer to the Radiation Therapy Treatment Room.

A variety of sizes and shapes of the pentagonal treatment cones were prepared so they fit the costal arch adequately and encompassed various anatomic situations of the tumor bed and the high risk lymph node groups noted above.

The field was clearly illuminated by an electric lamp fixed to a telescope attached to the treatment cone. The sterilized cone was inserted into the abdomen inclining degrees so the celiac axis was sufficiently covered.

Electronic energy and radiation dose of IORT

The electronic energy from 6 MeV to 16 MeV was selected. The radiation doses of IORT were selected according to the posibillity of the radicality achieved by surgical operation. A single dose of 10-15 Gy was given to 41 patients who had no clinically undetectable lesions, a single dose of 20 Gy was given to each 27 patients who were suspected to have microscopic residual LN, 25 Gy was given to 37 patients who were suspected to have macroscopic residual LN or direct invasion of adjacent structure, and 30 Gy to one patient who had noncurative surgery because of incomplete excision of metastatic lesions.

RESULTS

Complications

When IORT is used for gastric cancer, critical organs to which exposure must be avoided, are the pancreas, duodenum and jejunum which must be shielded from radiation. Less than 40% of the pancreas was generally included in the radiation field. Acute and late damage to the pancreas was evaluated by changes in serum amylase and blood glucose levels after IORT. Temporary increases in both serum amylase and blood glucose occurred after IORT, but they returned to preirradiation levels within a week. Neither significant late complications nor deviation

Table 2 The 5-yr survival rate of patients with operation combined with intraoperative radiotherapy (IORT)

Groups	*n*	Stage Ⅰ	Stage Ⅱ	Stage Ⅲ	Stage Ⅳ
IORT	106	13/13 (100%)	17/17 (100%)	29/48 (60.4%)	4/28 (14.3%)
Operation	441	65/70 (92.8%)	54/67 (80.6%)	110/22 (45.1%)	6/6 (10.0%)
P		> 0.10	< 0.001	< 0.005	> 0.05

Table 3 The 5-yr survival rate of patients with operation combined with intraoperative radiotherapy (IORT) in stage Ⅲ

Groups	*n*	D2 resection						D3 resection					
		n	1 yr	2 yr	3 yr	4 yr	5 yr	*n*	1 yr	2 yr	3 yr	4 yr	5 yr
IORT	48	9	100%	100%	87.5%	87.5%	60%	39	100%	100%	93.1%	85.5%	61%
Operation	244	114	81.6%	70.4%	52.3%	43.3%	35.7%	128	92.1%	85%	66.5%	62.2%	56.3%
P			< 0.05	< 0.001	< 0.001	< 0.001	< 0.005		> 0.50	> 0.10	< 0.001	< 0.005	> 0.05

from the usual postoperative course was observed. There was no instance of delayed wound healing. One patient died from cardiac infarction, resulting in a death rate of 0.9% (1/106). Recovery of gut function in all of the patients with IORT was delayed for 24 h.

Survival

Table 2 demonstrates the survival rates based on an analysis of 106 patients treated by IORT and 441 patients treated by operation alone. The survival rate was calculated by the Kaplan-Meier method.

The 5-year survival rate (YSR) for patients treated by operation alone was 92.8% for stage Ⅰ, 80.6% for stage Ⅱ, 45.1% for stage Ⅲ, and 10% for stage Ⅳ. On the other hand, the 5-YSR for patients treated by IORT was 100% for stage Ⅰ and stage Ⅱ, 60.4% for stage Ⅲ, and 14.3% for stage Ⅳ. As shown in Table 2, there was no difference between the 5-YSR of patients in stage Ⅰ, and stage Ⅳ in the two groups. The IORT procedure raised the survival of patients with stages Ⅱ and Ⅲ cancer from 15% to 20%, $P < 0.005$-0.001.

Table 3 demonstrates that the 5-YSR of the stage Ⅲ cancer patients with D2 radical resection combined with IORT was improved as compared with operation alone. The 5-YSR of the stage Ⅲ cancer patients with selective D3 radical resection combined with IORT was not improved as compared to an operation alone. However, the 3- and 4-YSR was significantly improved by IORT combined with operation as compared with operation alone. But the differences of the 3 and 4-YSR between the two groups were statistically significant.

DISCUSSION

As far as gastric adenocarcinoma is concerned, the prognosis remains poor with a high local failure rate estimated up to 67%[7]. While a lot of trials using systemic chemotherapy are currently being made to decrease the general failure rate in gastric adenocarcinoma, using IORT as a boost to EBRT could be a way to improve local control rate on coeliac area after gastrectomy[2,8]. IORT has been known as a feasible radiation treatment since 1907[9]. Its main advantage is probably to spare normal tissues while delivering a high dose precisely on the tumoral target. Extended lymph node dissection has been used mainly in Japan and the treatment results have improved remarkably as shown in many retrospective studies. But extended lymph node dissection is still controversial in the Western countries because of reported excessive postoperative morbidity and mortality and lack of proof in a recent retrospective study and in randomized controlled trials[1,10]. The Dutch Gastric Cancer Trial and the Medical Research Council (MRC) randomized surgical trial were not able to prove the benefit of extended lymph node dissection.

Due to operating room planning reasons, a great number of patients with gastric adenocarcinoma were not included in the IORT protocol in the reported study, and therefore, no valid comparisons or conclusions can be made. However, it is important to notice that mortality and morbidity rates in patients treated with IORT (1/42 and 3/42) were not excessive when compared with the mortality and morbidity rates reported in the retrospective study of 350 gastric cancer patients treated by surgery alone in the same institution since 1970 (respectively 10.8% and 14%)[11]. Glehen *et al*[12,13] did not detect any acute toxicity of IORT. Concerning the late toxicity of IORT, canine studies provide guidelines for human cancer treatment and the data provided by these canine studies are comparable with the data obtained in an autopsy study which validated the model for clinical use. With a long term follow-up, in the reported study we just detected one case of enteritis, 2 years after irradiation. But this late toxicity was controversial in the literature data because of the reported cases of other enteritis, gastrointestinal bleedings with or without arterioenteric fistulas, vertebral collapses and liver hemangiomas[2,14,15].

Each surgical procedure that does not achieve a complete local excision of the primary tumor in all 3 dimensions (length, width, aboral, and depth of tumor) and of the area of lymphatic drainage is, according to the UICC classification of D1 or D2 resection (i.e. a resection leaving residual microscopic or macroscopic tumor), considered to be a "palliative resection". In addition to procedures that leave the entire tumor *in situ*, any type of operation that leaves residual macroscopic or microscopic tumor at the resection margin, tumor bed, or in the

lymphatic drainage area must also be considered to be a palliative procedure.

The rationale for IORT is to increase the irradiation dose to the tumor without exceeding critical normal tissue tolerance. IORT used as a neoadjuvant therapy, can increase the 5-YSR of patients with advanced gastric carcinoma. Special linear accelerators have been used for the application of high single-dose radiation to the tumor bed[3,4]. Abe *et al*[5] reported 115 gastric cancer patients treated by IORT. The 5-YSR survival of patients in stages Ⅱ through Ⅳ treated by IORT increased by nearly 10%-20%. No difference in the survival of patients was observed between the two groups. IORT did not afford benefit if the lymph node metastases were limited with n_1 group or serosal invasion was not found. On the other hand, the 5-YSR for patients treated by IORT increased by nearly 10% when the serosal invasion was observed, and by nearly 18% when n_2 and n_3 lymph node metastases were found. Ogata *et al*[16] reported that the 5-YSR and 8-YSR for 58 cases treated by IORT were both 100% , and 60% and 48% in control group with stage Ⅱ cancer. The 8-YSR of the stage Ⅲ cancer by IORT was 55%, and 35% in the control group. But these results were controversial. Coquard *et al*[2] reported that the 5-YSR of patients with gastrectomy and local lymph node resection combined with IORT was the same as single expanded lymph node resection. However, the postoperative complications and mortality were lower than that in those who received only gastrectomy. Our results showed that operation combined with IORT is beneficial for patients in stage Ⅱ and stage Ⅲ cancer with a 5-YSR of 14.4%-20%. However, this procedure was ineffective for patients with stage Ⅰ and stage Ⅳ cancer treated with D2 lymphadenectomy combined with IORT, the 5-YSR of stage Ⅱ cancer patients was increased by 60% as compared with D2 lymphadenectomy only by 35.7%. With D3 lymphadenectomy combined with IORT, the 3- and 4-YSR were improved, but it was ineffective for the 1, 2 and 5-YSR. These encouraging results were already reported by Japanese authors[5,17] with a 10%-20% increase in 5-YSR in stages Ⅱ and Ⅲ. However, this has not been reported in Western countries, especially for pN+ patients. But in a shorter follow-up, a local recurrence of under 15% after the use of IORT and EBRT was reported[18,19].

The radiation target area in the region of the upper abdomen includes potential microscopic tumor extensions and known or suspected macroscopic disease[13]. In order to reduce the injury of irradiation to the normal tissues near the stomach, the target area is restricted to the gastric bed. The inferior field border is generally at the level of the L3 to L4 vertebrae, but this depends on the location of the carcinoma and the position of the stomach before treatment. The target area includes the proximal and regional lymphatics, the right and left cardiac nodes, the nodal chains of the lesser and greater curvatures, the suprapyloric and infrapyloric chains, the splenic chains, and the lymph nodes along the hepatic artery. For the cardiac tumors, upper extension of the field to the terminal esophagus and paraesophageal lymph nodes is recommended. If the tumor is unresectable or if, after surgery, macroscopic disease remains, the total irradiation dose can be increased in a coned-down volume, which is restricted to the area of gross disease. With regard to the opitimization of IORT, we developed a new technique that provides a wider irradiation field for patients undergoing total gastrectomy. In these patients, total gastrectomy, splenectomy, removal of the pancreas capsule, and extended lymph node dissection including the nodes along the splenic artery were performed. Then the tail and body of the pancreas were moved up to the right side margin of the abdominal aorta and the superior mesenteric vein. These maneuvers provided a much wider irradiation field, which included the para-aortic lymph nodes. After irradiation, the pancreas was returned to its normal position and an esophagoenterostomy was performed. This method was adopted only in patients without cancer invasion of the pancreas or metastases in the lymph nodes along the splenic artery. The advantage of this method is that it produces a wide irradiation field including para-aortic lymph nodes and does not increase the complications and mortality. Meanwhile, one must be careful to protect the duodenum and jejunum before irradiation as those organs must be shielded from the irradiation field.

With regard to special radiotherapeutic techniques that include the potential of relatively high biologically effective doses, gastric cancer is one of the most convincing indications for intraoperative irradiation. Special linear accelerators have been used for the application of high single doses (15-35 Gy) to the tumor bed. One problem with IORT is to correctly cover tumor sites outside the midline area or under the left hemidiaphgragm. In addition, the maximum tolerable dose administered in one session is limited from 15 to 35 Gy. Abe *et al*[5] reported that doses of irradiation depended on the radicality achieved by the operation. In general, the irradiation doses from 15-30 Gy were applied in curative situations and 30-35 Gy in palliative situations. We selected the doses of IORT according to the residual tumor volume, including clinically undetectable lesions (10-15 Gy), microscopic residual (20 Gy), macroscopic residual (25 Gy), or noncurative surgery (30 Gy). No serious complications occurred in these cases. Our data strongly support the efficacy of IORT for local tumor control.

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RAPID COMMUNICATION

Alterations of biliary biochemical constituents and cytokines in infantile hepatitis syndrome

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Abstract

AIM: To investigate the biliary biochemical constituents and cytokines in infantile hepatitis syndrome (IHS).

METHODS: From 42 IHS subjects and 21 controls, serum and biliary biochemical constituents, including total bilirubin (TBIL), direct bilirubin (DBIL), alanine aminotransferase (ALT), gamma-glutamyl transpeptidase (γ-GT), total bile acid (TBA), interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) both in bile and serum, were assayed. The subjects with IHS were divided into a cholestasis group (n = 21) and a hepatitis group (n = 21).

RESULTS: In the cholestasis group, serum TBIL, DBIL, ALT, γ-GT, TBA, IL-6 and TNF-α levels were higher than those in the control (P < 0.01); and also the biliary TBIL, DBIL, γ-GT and TBA levels were lower than those in the control, whereas biliary IL-6 and TNF-α levels were higher than those in the control (P < 0.01). In the cholestasis group, serum IL-6 and TNF-α levels were lower than those in bile (P < 0.01). In the hepatitis group, serum DBIL, ALT, γ-GT, TBA, IL-6 and TNF-α levels were higher than those in the control (P < 0.01 or 140.57 ± 70.32 *vs* 79.06 ± 35.25, P < 0.05), while biliary TBIL, DBIL, γ-GT and TBA levels were lower than those in the control (P < 0.01), and biliary IL-6 and TNF-α levels were higher than those in the control (P < 0.01). In the hepatitis group, serum IL-6 and TNF-α levels were also lower than those in bile (P < 0.01). Serum TBIL, DBIL, γ-GT, IL-6 and TNF-α levels in the cholestasis group were higher than those in the hepatitis group, while biliary IL-6 and TNF-α levels in the cholestasis group were higher than those in the hepatitis group. Biliary IL-6 and TNF-α were found to be more significantly increased than serum IL-6 and TNF-α in IHS (P < 0.01). The biliary IL-6 and TNF-α levels were positively correlated with serum DBIL, TBA and γ-GT levels in IHS subjects.

CONCLUSION: Biliary biochemical constituents alter in coincidence with pathological changes in hepatocellular injury. Cholestasis is more serious in IHS patients of cholestasis subtype. Assay of biliary IL-6 and TNF-α levels can be specific and sensitive to determine the inflammatory status of impaired liver in IHS.

Key words: Infantile hepatitis syndrome; Biliary biochemical constituents; Biliary cytokines; Interleukin-6; Tumor necrosis factor-α

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INTRODUCTION

Infantile hepatitis syndrome (IHS), with a morbidity of 1/2500 in live-born infants[1], comprises a series of symptoms, including jaundice, splenohepatomegalia, changes of texture of the liver, hepatic dysfunction in onset mainly in the neonatal period and infancy. It has been reported that the biochemical constituents and cytokines in blood alter when the disorder attacks[2]. However, what changes of those biochemical constituents and cytokines in bile can be, and what relationships between IHS and physiologic jaundice in those constituents can be, remains a puzzle in pediatric practice. In this study, we focused on the alterations of biochemical constituents and cytokines in serum and bile obtained from subjects attacked by IHS.

MATERIALS AND METHODS

Subjects

According to the diagnostic criteria[3], IHS was defined as: (1) age < 1 year; (2) jaundice; (3) splenohepatomegalia and changes of texture of the liver; and (4) alteration in hepatic function. Forty-two subjects [29 males and 13

Table 1 Comparison of serum and biliary biochemical constituents among three groups (mean ± SD, *n* = 21)

Group	TBIL (μmol/L)	DBIL (μmol/L)	ALT (nkat/L)	γ-GT (nkat/L)	TBA (μmol/L)
Serum					
Cholestasis	188.76 ± 72.88[b,d]	130.76 ± 48.18[b,d]	126.80 ± 82.12[b]	266.20 ± 92.73[b,d]	118.62 ± 43.58[b]
Hepatitis	125.91 ± 64.28	68.67 ± 36.75[b]	96.11 ± 53.69[b]	140.57 ± 70.32[a]	111.89 ± 51.54[b]
Control	124.89 ± 62.07	6.19 ± 2.55	24.23 ± 7.17	79.06 ± 35.25	14.50 ± 9.76
Biliary					
Cholestasis	78.13 ± 58.86[b]	52.63 ± 42.43[b]	8.07 ± 4.21	167.00 ± 100.06[b]	180.05 ± 216.98[b]
Hepatitis	89.95 ± 59.12[b]	63.56 ± 53.39[b]	8.21 ± 4.15	278.55 ± 201.10[b]	266.63 ± 268.09[b]
Control	252.36 ± 108.81	174.08 ± 92.56	7.22 ± 3.41	783.50 ± 363.28	643.63 ± 80.67

[a]$P < 0.05$ *vs* control; [b]$P < 0.01$ *vs* control; [d]$P < 0.01$ *vs* hepatitis group.

Table 2 Comparison of serum and biliary IL-6 and TNF-α among three groups (mean ± SD, *n* = 21, ng/L)

Group	Serum IL-6	Serum TNF-α	Biliary IL-6	Biliary TNF-α
Cholestasis	63.76 ± 26.67[b,d]	493.11 ± 137.26[b,d]	286.92 ± 102.02[b,d,f]	1625.89 ± 563.41[b,d,f]
Hepatitis	40.81 ± 19.32[b]	305.30 ± 94.45[b]	183.64 ± 76.94[b,f]	1068.55 ± 383.35[b,f]
Control	11.46 ± 6.54	214.32 ± 78.40	40.11 ± 19.89	535.80 ± 168.69

[b]$P < 0.01$ *vs* control group; [d]$P < 0.01$ *vs* hepatitis group; [f]$P < 0.01$ *vs* serum.

females; average age 56 d (range: 33-120 d)] suffering from IHS, being divided into cholestasis subtype ($n = 21$) and hepatitis subtype ($n = 21$) according to the color of stool[4], were randomly selected from in-patients in Wuhan Children's Hospital. Another 21 subjects [12 males and 9 females; average age 43 d (range: 23-68 d)], being ruled out from IHS and diagnosed as breast-feeding jaundice, were chosen as controls.

Methods

In fasting condition, next morning after admission to the hospital, non-anticoagulated venous blood was collected from all patients, and the blood specimens were examined instantly. The bile specimens were collected by infant duodenum drainage tube. The procedure of draining was carried out as mentioned below[5]. Before draining, the subjects were instructed to fast for 4 h and given intravenous transfusion for essential nutrition, and given diazapen intravenously or chloral hydrate orally if restless. Then, the infants were placed at right arm reclining and the head was retained by an assistant; after applying a small amount of liquid paraffin and dispelling the tampon at the end of the tube, the operator inserted the tube through right nasal cavity to the stomach at a depth of 30-35 cm and gastric juice was drained out; then through the pylorus, the tube accessed the duodenum at the depth of 40-45 cm and duodenal juice was drained out. The draining was considered successful if yellow draining juice was obtained, or the head of the draining tube was ascertained in the duodenum by X-ray.

The biliary and serum total bilirubin (TBIL), direct bilirubin (DBIL), alanine aminotransferase (ALT), gamma-glutamyl transpeptidase (γ-GT) and total bile acid (TBA) were assayed by CL-7200 Fully-automated Chemistry Analyzer provided by Shimadzu Co. Ltd. The biliary and serum interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) were assayed by double antibody Sandwich-ELISA with the apparatus from Micro Reader-3 and the kit from R&D Co. Ltd.

Statistical analysis

One-way ANOVA (*F*-test, SNK-test) was employed to determine the differences of data among the three groups. Student's *t* test was applied for the differences in same constituent between biliary and serum data. Pearson correlation analysis was adopted to explore the relationships among the data. Statistical analyses were performed using SPSS 12.0 software.

RESULTS

Comparison of serum biochemical constituents and cytokines among three groups

Serum TBIL, DBIL, ALT, γ-GT, TBA, IL-6 and TNF-α levels were significantly higher in the cholestasis group than those in controls ($P < 0.01$). Moreover, serum DBIL, ALT, γ-GT, TBA, IL-6 and TNF-α levels were markedly higher in the hepatitis group compared to the controls ($P < 0.01$ or $P < 0.05$); while there was no significant difference in serum TBIL level between the hepatitis and control group. In addition, serum TBIL, DBIL, γ-GT, IL-6 and TNF-α levels in the cholestasis group were markedly higher than those in the hepatitis group ($P < 0.01$), but ALT and TBA levels were not obviously different between these two groups (Tables 1 and 2).

Comparison of biliary biochemical constituents and cytokines among three groups

Biliary TBIL, DBIL, γ-GT and TBA levels both in cholestasis and hepatitis groups were significantly lower than those in controls ($P < 0.01$), while biliary IL-6 and TNF-α levels in those two groups were notably higher

than those in controls ($P < 0.05$). In contrast to that in the hepatitis group, the value of biliary IL-6 and TNF-α in cholestasis group markedly increased ($P < 0.01$), but TBIL, DBIL, γ-GT and TBA levels in both groups were not significantly different. However, the value of biliary ALT was not obviously different among the three groups (Tables 1 and 2).

Comparison between biliary and serum cytokines

Both in cholestasis and hepatitis groups, the levels of IL-6 and TNF-α in serum were notably lower than those in bile ($P < 0.01$) (Table 2).

Relationships between biochemical constituents and cytokines

Biliary IL-6 was obviously correlated with serum DBIL, ALT and γ-GT with the coefficient of correlation of 0.4621, 0.4152 and 0.5376, respectively ($P < 0.05$). Moreover, the biliary TNF-α was significantly correlated with serum DBIL, ALT and γ-GT with the coefficient of correlation of 0.3972, 0.4309, 0.4713, respectively ($P < 0.05$). However, the serum IL-6 and TNF-α were not correlated with serum TBIL, DBIL, ALT, γ-GT, and TBA.

DISCUSSION

Serum biochemical constituents, such as TBIL, DBIL, ALT, γ-GT and TBA, are used to monitor liver function in medical practice. Among those constituents, bilirubin is a breakdown product of hemoglobin, and total and direct bilirubin (TBIL and DBIL) are usually measured to screen for or to monitor jaundice caused by liver or gall bladder dysfunction. Alanine aminotransferase (ALT), an enzyme found mainly in the liver, is released into the bloodstream when the liver is damaged or diseased. Gamma-glutamyl transpeptidase (γ-GT), existing in the endochylema of the hepatocyte and epithelium of the intrahepatic bile duct and being mainly synthesized by mitochondria in hepatocytes, is discharged to the duodenum through bile duct. Therefore, rise of γ-GT indicates hepatocytic dysfunction or obstruction of extra-hepatic bile duct. Especially, detection of biliary γ-GT can differentiate extra-hepatic biliary atresia and IHS, for bile not consisting of γ-GT when biliary atresia occurs[5]. Total bile acid (TBA) is an exclusive index reflecting hepatic synthesis, secretion, metabolism and hepatocellular dysfunction, and it has been shown that its specificity, sensitivity and stability tendency in numerical hepatobiliary disorder in liver are all superior to the conventional liver function examinations[6].

Cytokines are small proteins released by cells that have a specific effect on interactions and communications between cells or on behavior of cells, and participate in many pathophysiologic progresses in hepatobiliary disorders. Biliary cytokines are produced by hepatocytes, macrophages and epithelium of bile duct. It has been reported that biliary IL-6 was exclusive for diagnosis of angiocholitis[7] and TNF-α could reflect the extent of angiocholitis[8]. We previously confirmed that under cholestatic condition, biliary IL-6 and TNF-α levels increased and were correlated with hepatocellular impairment and cholestasis in rabbit, thereby indicating that IL-6 and TNF-α could reflect the extent of hepatocellular necrosis and angiocholitis[9].

In this study, we observed that, compared to the controls, almost all biochemical constituents in serum were remarkably increased both in cholestasis and hepatitis groups, while those constituents in bile were mostly decreased, which indicated discharging of those from liver to blood and reduction of those in bile when hepatocyte inflamed and cholangiole was embarrassed. When compared between cholestasis and hepatitis groups, the cholestasis-related indexes TBIL, DBIL and γ-GT in serum increased more obviously in the cholestasis group than those in the hepatitis group, while no significant differences in those biochemical constituents in bile between these two groups were observed. In addition, the ALT and TBA, the indexes related to hepatocellular impairment, were not markedly different between the two groups both in serum and bile, manifesting the feature of cholestasis in IHS.

Following the previous animal experiment, we examined the two related cytokines to explore the role of IL-6 and TNF-α in IHS. We found that the two cytokines notably rose in blood and bile of IHS subjects compared to the controls, thereby indicating the inflammatory status in IHS. Furthermore, IL-6 and TNF-α in the cholestasis group increased more significantly than that in the hepatitis group, elucidating the greater severity in cholestasis. On the other hand, in both cholestasis and hepatitis groups, biliary cytokines were more elevated than serum cytokines, which implied biliary cytokines could be a more sensitive clue for diagnosing hepatic impairment.

From the statistical analysis, it was confirmed that biliary IL-6 and TNF-α had a positive correlation with serum DBIL, ALT and γ-GT, but the serum cytokines had not any correlations with serum biochemical constituents. This result illuminated that as cytokines in blood could be influenced by the state of whole body, assaying biliary inflammatory cytokines might be a specific and sensitive test for monitoring the development of IHS. Thus, the results clearly revealed that biliary biochemical constituents altered in coincidence with pathological changes in hepatocellular injury, which can demonstrate the severity of IHS, especially for cholestasis, and the differences between cholestasis and hepatitis subtype in this disorder. Furthermore, it can be concluded that the test of biliary IL-6 and TNF-α might be a specific and sensitive reference to determine the inflammation status of the impaired liver in IHS.

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RAPID COMMUNICATION

Construction of an oral recombinant DNA vaccine from *H pylori* neutrophil activating protein and its immunogenicity

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Abstract

AIM: To construct a live attenuated *Salmonella typhimurium* (*S. typhimurium*) strain harboring the *H pylori* neutrophil activating protein (HP-NAP) gene as an oral recombinant DNA vaccine, and to evaluate its immunogenicity.

METHODS: By genetic engineering methods, the genomic DNA of *H pylori* was extracted as a template. The total length of the HP-NAP gene was amplified by polymerase chain reaction (PCR) and cloned into pBT vector for sequencing and BLAST analysis, then subcloned into a eukaryotic expression vector pIRES followed by PCR identification and restriction enzyme digestion. The identified recombinant plasmid pIRES-NAP was transfected into COS-7 cells for target fusion protein expression, and its antigenicity was detected by Western blotting. Then the recombinant plasmid was transformed into a live attenuated *S. typhimurium* strain SL7207 as an oral vaccine strain, and its immunogenicity was evaluated with animal experiments.

RESULTS: A 435 bp product was cloned using high homology with HP-NAP gene in GenBank (more than 98%). With identification by PCR and restriction enzyme digestion, a recombinant eukaryotic expression plasmid pIRES-NAP containing the HP-NAP gene of *H pylori* was successfully constructed. The expressed target protein had a specific reaction with *H pylori*i whole cell antibody and showed a single strip result detected by Western blotting. Oral immunization of mice with recombinant DNA vaccine strain SL7207 (pIRES-NAP) also induced a specific immune response.

CONCLUSION: The successful construction of HP-NAP oral DNA vaccine with good immunogenicity may help to further investigate its immunoprotection effects and develop vaccine against *H pylori* infection.

INTRODUCTION

The discovery of *H pylori* has brought about a revolution in the research of etiological factors of gastrointestinal diseases[1]. It has been confirmed that *H pylori* is the main cause of chronic superficial gastritis, chronic active gastritis and peptic ulcer[2-4], and has a close relation to gastric mucosa-associated lymphoid tissue lymphoma and gastric cancer[5,6]. In 1994, the World Health Organization defined it as a class 1 carcinogen. Although significant progress has been made in treating *H pylori* infection with current triple or quadruple therapy based on antibiotics and proton pump inhibitors, the limitations of pharmacological therapy such as side effects, poor compliance, high cost, and most importantly, rapid emergence of antibiotic resistance have set the stage for the development of less costly and more efficient means to prevent and control *H pylori* infection. Ample precedence from previous experiences suggests that vaccination may be an alternative[7].

DNA vaccine has shown a great potential in protecting against and treating many diseases since it was developed. It can induce complete immune responses, provide heterologous cross protection, and can be easily prepared as a polyvalency vaccine[8]. In addition, the live attenuated *Salmonella typhimurium* (*S. typhimurium*) strain expressing foreign antigens may be a very hopeful new-generation for developing *H pylori* vaccine. Experiments on human body indicate that it has very good endurance and immunogenicity, which can be used to transmit foreign antigens[9]. In our present study, we selected a neutrophil activating protein (HP-NAP), a new major virulence factor of *H pylori* identified more recently, which was termed for its ability to induce adhesion of neutrophils to gastric endothelial cells and to produce reactive oxygen radicals[10]. We attempted to construct a live attenuated

S. typhimurium strain harbouring the HP-NAP gene as an oral recombinant DNA vaccine, and to explore its immunogenicity to pave the way for biological treatment of *H pylori* infection.

MATERIALS AND METHODS

Materials

The *H pylori* standard strain CCUG 17874, kindly presented by the IRIS Research Center of Italy, was cultured on *Campylobacter* selective agar (Merck, Germany) medium supplemented with 10% defibrillated goat blood containing *Campylobacter* selective antibiotic mixture (Merck), and incubated under microaerobic conditions (50 mL O_2, 85 mL N_2, 10 mL CO_2 and 10% relative humidity at 37℃). The *E. coli* strain DH5α, live attenuated *S. typhimurium* strain LB5000 and SL7207, and COS-7 cell lines conserved in our laboratory, were cultured routinely.

Restriction enzymes including *Xho* I and *Mlu* I, T4 DNA ligase, and *EX Taq*™ DNA polymerase were purchased from TaKaRa Biotechnology (Dalian) *Co*., *Ltd*. Mouse anti-HP-NAP antibody was prepared by our laboratory. Alkaline phosphatase anti-mouse IgG made in horse was purchased from Vector Laboratories. Lipofectamine™ 2000 was purchased from Gibco Corporation. T-A cloning vector pBT was purchased from Shanghai Sangon Biological Engineering & Technologies and Services *Co*., *Ltd*. Eukaryotic expression vector pIRES was purchased from Clontech of BD Biosciences. Other reagents were analytically pure reagents produced in China.

Twenty-four specific-pathogen free male C57BL/6 mice aged 4 wk, were purchased from Sino-British Sippr/Bk Laboratory Animal *Ltd*. of Shanghai.

Construction of recombinant plasmid pIRES-NAP

According to the nucleotide sequence of the HP-NAP gene in GenBank, we designed a pair of oligonucleotide primers P1 (5′-GTC CTC GAG ATG AAA ACA TTT GAA ATT TTA AAA CAT TTG CAA GCG-3', with *Xho* I restriction site) and P2 (5′-GTC ACG CGT TTA AGC CAA ATG GGC TTG CAA CAT CC-3', with *Mlu* I restriction site) synthesized by Sangon with correct ORF. Genomic DNA of CCUG 17874 was extracted as the template. Four mL of template DNA was added to a 100 mL reaction mixture containing 10 mL 10 × PCR buffer, 0.2 mmol/L each deoxynucleoside triphosphate, 2.5 U of *EX Taq*™ polymerase, and 0.2 mmol/L each primer. PCR was performed with Mastercycler® gradient thermocycler (Eppendorf, Germany) as follows. The initial denaturation cycle was at 94℃ for 5 min, followed by 30 cycles of denaturation at 94℃ for 1 min, primer annealing at 60℃ for 1 min, and DNA chain extension at 72℃ for 1 min. The final cycle was at 72℃ for 10 additional minutes, followed by rapid cooling to 4℃. The purified PCR products were T-A cloned into pBT vectors, then transformed into DH5α competent cells using standard methods. Positive clones were screened by blue/white spot and ampicilin resistance. Single-stranded DNA was prepared from selected clones for sequencing with forward M13 universal primer. Homologous analysis between the cloned HP-NAP gene and related genes in GenBank for neucleotide and deduced amino acid sequence was performed by BLAST. The identified recombinant plasmid pBT-NAP was digested with restriction endonucleases *Xho* I, *Mlu* I and subcloned into the corresponding sites of eukaryotic expression vector pIRES. PCR with primers P1, P2 and double enzyme restriction were performed.

Assay of HP-NAP fusion protein expression

COS-7 cells were cultured routinely and inoculated into 6-well plates one day before transfection, then co-cultured with a mixture of Lipofectamine™ 2000 and recombinant plasmid pIRES-NAP mixed instantly in definite proportion. After incubation at 37℃ for 24-48 h, the culture was centrifuged to collect supernatant. Western blot analysis was performed to evaluate the immunity of HP-NAP antigen expressed in culture supernatant using mouse anti-HP-NAP as primary antibody and horse anti-mouse IgG as secondary antibody.

Construction of oral DNA vaccine

Recombinant plasmid pIRES-NAP was transformed into *S. typhimurium* strain LB5000 for methylation decoration, then extracted and transformed by electroporation into ending host bacteria *S. typhimurium* strain SL7207. SL7207 (pIRES-NAP) was grown in LB medium containing 100 mg/mL ampicilin at 37℃ for 60 generations. Identification by PCR amplification and double restriction endonuclease digestion was performed every 10 generations.

Assay of immunology of oral vaccine

Twenty-four mice were divided into 3 groups (8 mice in each group). LB group consisting of non-immunized mice received LB culture fluid and was used as a control group, *Salmonella* group was immunized merely with attenuated The *S. typhimurium* strain SL7207, the vaccine group was immunized with recombinant strain SL7207 (pIRES-NAP). Prior to immunization, all the mice were left overnight without solid food and 6 h without water. A total volume of 100 μL of 30 g/L sodium bicarbonate was given orally using a special catheter to neutralize the stomach pH. Then the mice in the LB group were lavaged immediately with 200 μL LB fluid. Mice in the *Salmonella* group and vaccine group were lavaged with 1.0×10^9 c.f.u of *S. typhimurium* strain SL7207 and vaccine strain SL7207 (pIRES-NAP), respectively, in a total volume of 200 μL. At 4 wk after immunization, the mice were sacrificed by terminal cardiac puncture under anesthesia and small intestine juices were collected. Indirect ELISA was performed to evaluate HP-NAP-specific IgG or IgA in serum and intestine juice. Purified HP-NAP was used as a coating antigen in ELISA immunoassay.

RESULTS

PCR amplification and homology analysis of HP-NAP gene

A 435 bp gene segment was amplified by PCR, which was consistent with the complete sequence of the HP-NAP gene confirmed by sequencing result (Figure 1). BLAST analysis showed that the nucleotide homology between cloned HP-NAP gene and *H pylori* SS1 strain from Genbank reached 98.2% (427/435), including 3 of C-T

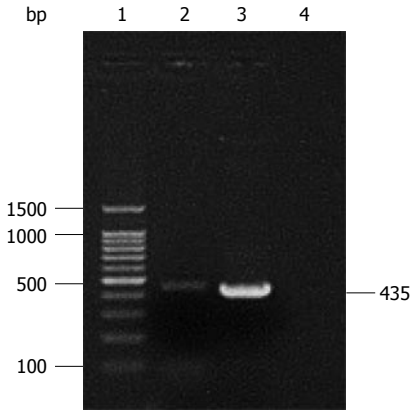

Figure 1 Electrophoresis of HP-NAP PCR products. Lane 1: 100 bp DNA ladder marker; lanes 2 and 3: PCR products of HP-NAP; lane 4: Blank control.

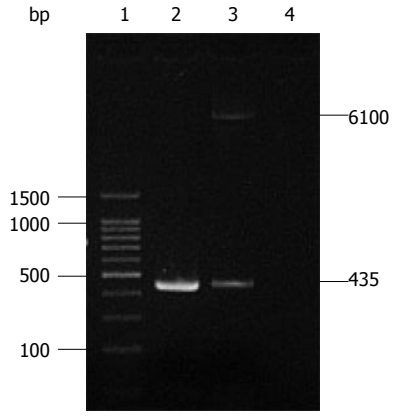

Figure 2 Identification map of recombinant plasmid pIRES-NAP. Lane 1: 100 bp DNA ladder marker; lane 2: PCR products templated on pIRES-NAP; lane 3: pIRES-NAP digested by endonucleases Xho I and Mlu I; lane 4: Blank control.

and 4 of A-G replacements, and more than 97% (426/435, 425/435) of other common *H pylori* strains such as 5D, 5A, 2B, 2A, RHP901a, DB2 and 1811a. Base replacements of cloned sequence did not significantly affect the translating results. The homology between proteins translated by cloned gene and SS1 strain was 98.6% (142/144).

Identification of recombinant plasmid

A 435 bp target product was cloned as a template on the recombinant plasmid pIRES-NAP using primers P1 and P2. *Xho* I and *Mlu* I enzyme digestion also revealed the target HP-NAP gene in plasmid pIRES-NAP (Figure 2), suggesting that the recombinant plasmid pIRES-NAP and the oral DNA vaccine strain SL7207 (pIRES-NAP) were successfully constructed.

Assay of HP-NAP fusion protein

Western blot analysis results of culture supernatant of COS-7 cells transfected with recombinant plasmid pIRES-NAP are shown in Figure 3. A special simple strip about 17 000 in relative molecular weight was obtained from the supernatant of transfected COS-7 cells, corresponding to the presumed consequence, but no strip was found in the supernatant of non-transfected COS-7 cells as control.

Immunology of recombinant strain

The sera and intestinal juices of the vaccine group immunized with SL7207 (pIRES-NAP) showed positive ELISA results while those of the LB group and *Salmonella* group showed negative ELISA results, indicating that *S. typhimurium* SL7207 (pIRES-NAP) could enable the organism to generate specific mucosal and humoral immunity against the HP-NAP antigen.

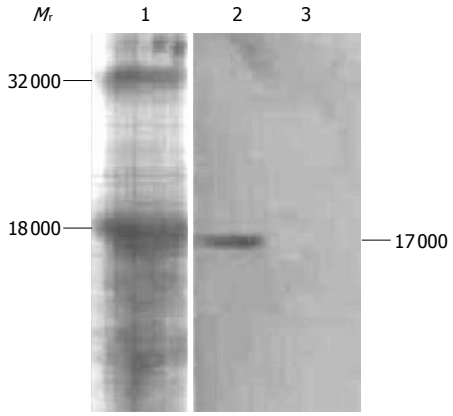

Figure 3 Western blot analysis of HP-NAP fusion protein expression. Lane 1: Protein standard; lane 2: COS-7 cells transfected with pIRES-NAP; lane 3: COS-7 cells without transfection as control.

DISCUSSION

Research on the *H pylori* vaccine has been mainly focused on the development of protein vaccines during the past decades, but the preparation and purification of protein antigens is time-consuming and laborious. Since effective immune responses rely on the presence of adjuvants, most of which are toxic to the organism, it is therefore important to develop new *H pylori* vaccines.

Recent advances in immunology and molecular biology have permitted development of DNA vaccines, which have a wide range of applications. Vaccines for such diseases as HIV infection, malaria, and tuberculosis are being developed by using plasmid DNA or viral or bacterial vectors to deliver the genes encoding antigens from pathogens to the host[11]. As live attenuated virus vaccines have come into being for decades, antigenic proteins can be produced *in situ* by the host, engendering cellular and humoral immune responses. But unlike live attenuated vaccines, gene-based vaccines are being designed to deliver only the genes encoding the antigens for the vaccine. The ability of gene-based vaccines to generate cellular as well as humoral responses may be crucial in developing effective vaccines against *H pylori*-induced diseases. Similarly, the ability of gene-based vaccines to generate certain forms of immunogen such as a protein with a particular structure that can be formed only by mammalian cells *in situ*, may be a critical feature of DNA vaccines. It is thought that DNA vaccines can be produced and distributed on a global scale for prevention of diseases such as HIV infection, malaria, and tuberculosis.

S. typhimurium can be phagocytized by macrophages and M cells in Peyer node and through mesentery lymph nodes reach the liver and spleen, further stimulating other

organs and tissues to develop mucous membranes, cell and body fluid immunization responses. In the past few years, attenuated *S. typhimurium* as a delivery system has become a new trend to study a new type of oral recombinant live vaccine. Compared with traditional vaccines, live attenuated *S. typhimurium* is used as a new type of vector releasing system for heterologous antigens which does not require antigen purification, and not only protects antigens from degradation and denaturation in the stomach but also expresses adjuvant activity and prevents oral tolerance[12,13].

The development of *H pylori* DNA vaccine is unfolding. Todoroki *et al*[14] have investigated the effect of DNA vaccines encoding *H pylori*-heat shock proteins A and B (pcDNA3.1-hspA and -hspB) on inducing immune responses against *H pylori* in mice. In their study, C57BL/6 mice aged 5 wk were immunized by a single injection of 10 mg of pcDNA3.1-hspA and pcDNA3.1-hspB into intracutaneous tissue. Plasmid DNA lacking the inserted Hsp was injected as a control. Their results demonstrated that DNA vaccines encoding *H pylori*-Hsp could induce a significant immune response against *H pylori* and decrease gastric mucosal inflammation. Miyashita and his colleagues[15] reported that both intranasal and intracutaneous vaccination with pcDNA3.1 encoding *H pylori*-catalase (kat) induces humoral immune responses and suppresses *H pylori* colonization and inflammation of gastric mucosa. Serum IgG and IgA antibodies were induced in mice immunized with intracutaneous pcDNA3.1-kat with suppressed *H pylori* colonization compared to the mice immunized with control DNA, indicating that an effective DNA vaccine can be a new approach against *H pylori* infection in humans with potential foreground.

HP-NAP, a 150 000 dodecameric protein, is released in the medium, most likely after cell lysis, and binds to the bacterial surface, where it acts as an adhesion, mediating its binding to mucin[16] or to polymorphonuclear leukocyte sphingomyelin[17]. Purified recombinant HP-NAP is chemotactic for human neutrophils and monocytes[10] and induces surface expression of β_2-integrins which are necessary for endothelial trans-migration[18], suggesting that HP-NAP plays a role in the accumulation of these cells at the *H pylori*-infected site. HP-NAP is also a powerful stimulant of the production of reactive oxygen radicals and acts via a cascade of intracellular activation events, including increase in cytosolic calcium ion concentration and phosphorylation of proteins, leading to the assembly of functional NADPH oxidase on neutrophil plasma membrane through a pertussis toxin-sensitive pathway involving extracellular-regulated kinase (ERK) and p38-mitogen-activated protein kinase (MAPK)[19]. The activation of ERK and p38-MAPK is essential for the HP-NAP-induced superoxide anion generation, adhesion and chemotaxis of human neutrophils. Cytokines such as TNF and IFN-γ are reported to enhance the production of ROIs induced by HP-NAP and increase the damage of gastric mucosa[20].

HP-NAP has also been shown to increase the synthesis of tissue factor and the secretion of inhibitor-2 of the plasminogen activator in mononuclear cells. By inducing the coordinate expression of cell pro-coagulant and antifibrinolytic activities, HP-NAP might favor fibrin deposition and contribute to the inflammatory reaction of gastric mucosa elicited by *H pylori*[21]. HP-NAP is also capable of crossing epithelial monolayers and inducing activation of the underlying mast cells[22]. These data further support the idea that HP-NAP has an important role in the *in vivo* triggering and maintaining of inflammatory events observed during *H pylori* infection. Once released from the bacterium, HP-NAP would traverse the stomach epithelial layer, reaching the underlying tissue where mast cells reside. The subsequent activation of mast cells by HP-NAP with release of the content of the granules and pro-inflammatory cytokine IL-6, is known to recruit monocytes and neutrophils. Thus, HP-NAP can act at different stages of the inflammatory response by activating mast cells with release of pro-inflammatory molecules able to activate neutrophils and monocytes, and additionally by acting directly on neutrophils and monocytes by promoting their recruitment and activation.

HP-NAP is highly immunogenic in humans. Analysis of serum samples from 35 *H pylori*-infected individuals revealed that 60% of the subjects contain specific antibodies to HP-NAP[2]. Satin and his colleagues[10] immunized 10 mice with purified recombinant HP-NAP by intragastric administration, showing that 80% of them have acquired protective immunity, which is higher than the CagA (70%) group but lower than the supersonic lysate group (90%). These data indicate that this protein is a good vaccine candidate for protecting *H pylori* infection.

In the present study, we successfully amplified and subcloned the HP-NAP gene into the eukaryotic expression vector pIRES, and established the recombinant live attenuated *S. typhimurium* strain SL7207 (pIRES-NAP) as an oral DNA vaccine. BLAST analysis indicated the HP-NAP gene we cloned had a high homology with those in GenBank. The COS-7 cells transfected with recombinant plasmid pIRES-NAP expressing a protein of 17 000 reacted especially with anti-HP-NAP antibody, but no reaction was found in the control group. On the other hand, the serum and intestine fluids from mice immunized with DNA vaccine contained specific antibody to HP-NAP, while those from control groups did not.

In conclusion, HP-NAP DNA vaccine can express target protein with good immunogenicity in eukaryotic hosts, and *S. typhimurium* strain SL7207 (pIRES-NAP) may be a good candidate as a vaccine for prevention and cure of *H pylori* infection. Further study is needed to explore its immunoprotection effects.

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RAPID COMMUNICATION

Randomized clinical trial on seven-day-per-week continuous accelerated irradiation for patients with esophageal carcinoma: Preliminary report on tumor response and acute toxicity

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Key words: Esophageal carcinoma; Continuous accelerated irradiation; Conventional irradiation; Toxicity; Overall treatment time

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Abstract

AIM: Tumor response and normal tissue toxicity of seven-day-per-week continuous accelerated irradiation (CAIR) for patients with esophageal carcinoma were evaluated and compared to conventional irradiation (CR).

METHODS: Sixty patients with squamous cell carcinoma of the esophagus were randomized into two groups: the CAIR group (30 patients) and the CR group (30 patients). Patients in the CAIR group received radiotherapy (RT) with 2 Gy/fraction per day at 7 d/wk with a total dose of 50-70 Gy (average dose 64.2 Gy). The overall time of irradiation was 3.6-5.0 wk (average 4.6 wk). RT in the CR group was 2 Gy/fraction per day at 5 d/wk with a total dose of 40-70 Gy (average dose 61.7 Gy). The overall time of irradiation was 4.0-7.0 wk (average 6.4 wk).

RESULTS: The data showed that the immediate tumor response to RT was better in the CAIR group than in the CR group. Efficiency rates (CR plus PR) were 82.8% (24/29) and 58.6% (17/29), respectively (P = 0.047). In both groups the incidences of esophagitis and tracheitis were insignificant (P = 0.376, 0.959), and no patient received toxicity that could not be tolerated.

CONCLUSION: CAIR shortens overall treatment time and is well tolerated by patients. It may be superior to CR in enhancing the local response of tumor, but its remote effect for esophageal carcinoma awaits further follow-up.

INTRODUCTION

Esophageal carcinoma, especially in China, is one of the most common cancers. Its treatment results are rather dismal, with 5-year survival rates of about 5%-10% for conventional radiotherapy (CR)[1,2]. The poor prognosis is the result of both, local residual disease and early disease relapse. Thus for esophageal carcinoma, local control is the most important factor in prolonging survival[3]. Several animal experiments and clinical investigations have shown that accelerated repopulation of surviving tumor clonogens during a standard course of RT is one of the major reasons for treatment failure in several cancers[4-6]. Some clinical trials of accelerated hyperfractionated RT have been carried out with the aim of overcoming this problem by shortening the overall treatment time. Some reports have already suggested improvement in local control and survival rates, but at the expense of increasing acute toxicity, particularly with the faster schedule[6-8]. In recent years, another RT schedule has been used to treat head and neck cancer. The idea was simple-to continue radiation during the weekends. In this way, the overall treatment time has shortened for about two weeks, giving one fraction per day, seven days a week (including Saturday and Sunday), without any change of the other parameters as time or dose. This schedule was defined as a continuous accelerated irradiation (CAIR) and has been compared to conventional five days treatment in a randomized prospective study for head and neck cancer[9]. Using this RT schedule, patients with esophageal carcinoma were treated and our study aimed to evaluate tumor response and normal tissue toxicity.

MATERIALS AND METHODS

Materials

From October 2003 to December 2005, 60 unresectable or medically inoperable patients with esophageal carcinoma were enrolled and randomized into two groups by the sealed envelope method. The project of clinical randomized trial on seven-days-per-week continuous accelerated irradiation (30 patients) *vs* conventional treatment (30 patients), including the criteria for patient eligibility, the diagnostic procedure, the randomization method, the fractionation schemes of treatment techniques, and patient care was approved by the Ethical Committee of Xuzhou Cancer Hospital. All patients received full information concerning the aim of the study, diagnostic and treatment procedures, medical care, risk of acute and late sequelae before they entered the trial. All patients gave informed consent to this study.

Only patients with histologically proven squamous cells of esophageal carcinoma were included in the trial. Additional criteria for eligibility were age $\leqslant$ 75 years, Karnofsky performance status $\geqslant$ 70, white blood cell and hemoglobin levels within normal range, and no prior treatment. The pretreatment evaluation generally included chest radiography, chest CT scan, esophageal barium film, and ultrasound examination of the abdomen, including liver, kidney, spleen, and retroperitoneal lymph nodes, and liver and renal function tests. Based on examinations mentioned above, tumor staging was performed according to the TNM staging system of the 1997 American Joint Committee on Cancer staging system. Patients' characteristics are presented in Table 1 which shows comparable distribution of biological and clinical factors in both groups of the trial.

Table 1 Patients' characteristics

Characteristic	CAIR group	CR group	χ^2 or t	P
n	30	30		
Gender			0.30	0.584
Male	19	21		
Female	11	9		
Age (yr)	66.0 ± 8.0	70.9 ± 9.4	1.781	0.083
Length (cm)	3-10	2-12		
Average (cm)	5.5 ± 1.8	6.2 ± 3.1	0.962	0.342
Location			0.018	0.985
Upper-thoracic	4	6		
Middle-thoracic	23	19		
Lower-thoracic	3	5		
Stage			1.920	0.089
Ⅰ	0	2		
Ⅱ	17	12		
Ⅲ	13	16		
WBC ($\times 10^9$/L)	6.4 ± 1.9	6.1 ± 2.0	0.443	0.660
HGB (g/L)	138.4 ± 14.4	131.4 ± 17.1	1.411	0.166

Methods

Radiation methods: Radiation source was 6MV-X-ray linear accelerator. For the design of the radiation fields for all patients, a three-field approach was used: one anterior and two posterior oblique portals.The length of the field should cover clinical tumors with 3 cm extended margin at both ends of the lesion. The width of the fields was adjusted to cover gross tumors with 2 cm margins to include the subclinical lesions. RT in CAIR group was 2 Gy/fraction per day for 7 d/wk with a total dose of 50-70 Gy (average dose 64.2 Gy), the overall time of irradiation was 3.6-5.0 wk (average 4.6 wk). RT in CR group was 2 Gy/fraction per day for 5 d/wk with a total dose of 40-70 Gy (average dose 61.7 Gy), the overall time of irradiation was 4.0-7.0 wk (average 6.4 wk).

Tumor response and acute radiation reactions evaluated: All patients received esophageal barium examination before, during, and at the end of RT. At the end of RT, the tumor response to RT was evaluated. A complete response (CR) was the disappearance of the mass shadow, no narrowing observed in the esophageal lumen, and no, or slight rigidity of the esophageal wall remains without residual ulceration. A partial response (PR) was a > 50% reduction in tumor bulk but < 100% resolution of the disease and a residual shallow ulcer with a diameter of < 1.5 cm, despite the disappearance of the mass shadow. A minor response (MR) was definite improvement in the barium esophagogram but with < 50% regression, with a large residual ulcer crater and/or narrowing of the esophageal lumen, regardless of the residual state of the mass shadow. No change (NC) was no improvement in the X-ray findings, with a deep and large residual ulcer or complete obstruction of the esophageal lumen, regardless of the residual state of the mass shadow[10]. Acute radiation toxicity was evaluated by the Radiation Therapy Oncology Group (RTOG) toxicity criteria.

Statistical analysis

Statistical analysis was done by SPSS (Version 10.0). t-test, Chi-square test, or Wilcoxon-W test were used to compare the patients' characteristics, tumor response, and normal tissue toxicity to RT of both groups.

RESULTS

Early tumor response

One patient in the CAIR group interrupted RT because of multi-metastasis and another patient in CR group died from cardiac muscle infarction in the schedule. Within three months after RT, the patients that completed the schedule planed were evaluated by criteria as described above. In the two groups, efficiency rate (CR plus PR) was 82.8% (24/29) and 58.6% (17/29), respectively and, accordingly, the inefficiency rate (MR plus NC) was 17.2% (5/29) and 41.4% (12/29), respectively. The difference in tumor response to RT was statistically significant, the efficiency rate in the CAIR group was higher than the CR group (P = 0.047). The immediate response of the two groups of patients to RT are listed in Table 2.

Acute radiation reactions

Table 3 shows the acute radiation reactions during the treatment course and up to three months after RT. We found that acute radiation esophagitis and tracheitis in both groups was mainly grade Ⅰ-Ⅱ and the difference between the two groups was not statistically significant. No patient received treatment resulting in intolerable acute

Table 2 Tumor response to RT *n* (%)

Group	CR	PR	MR	NC	χ^2	*P*
CAIR	8 (27.6)	16 (55.2)	4 (13.8)	1 (3.4)	4.08	0.047
CR	6 (20.7)	11 (37.9)	12 (41.4)	0		

radiation reactions in either group.

Impact on blood cell and hemoglobin level

In the CAIR group, the total number of white cells declined below normal level in 5 patients within two weeks after beginning RT. In three of them, white cell counts normalized by medical intervention. In three patients of the CR group, total number of white cell declined below normal level. In both groups, there were two whose patients, total number of white cells did not increase right up to the end of RT. Furthermore, all patient's hemoglobin level had no statistically significant change during the treatment course.

DISCUSSION

Esophageal carcinoma is one of most common malignant diseases in China. The prognosis for patients with esophageal carcinoma is extremely poor. The five years survival rate is 5%-10% for CR alone. The poor prognosis is the result of both local treatment failure, seen in up to 80% of cases, and early disease dissemination[3,10]. Thus, for esophageal carcinoma, local control is at present the most important factor in prolonging survival. Accelerated repopulation of tumor cells during conventionally fractionated radiotherapy is a proposed reason of failed local control in head-and-neck tumors. In the clinical setting, one goal of treatment is to limit the extent of tumor cell regeneration that occurs during a course of fractionated RT. There is radiobiological rationale and convincing evidence from a number of clinical studies that a therapeutic gain may be achieved, at least for head and neck cancers, when conventional fractionation is modified by reduction in size of dose per fraction with the increase in total dose, reduction of overall treatment time, or both[9,11]. Rapid repopulation of tumor clonogens is able to compensate about 0.6 Gy/d[5], beginning after a lag period, which on average, in head and neck tumors is about 3-4 wk from the inception of therapy[12]. Thus, shortening overall treatment time should limit the extent of accelerated tumor repopulation, and therefore one may expect an increase in the probability of tumor control for given total dose. Since treatment time is thought to have little or no influence on the response of late reacting normal tissue, a reduction in overall treatment time would not be expected to affect the incidence and severity of late normal tissue injury (provided the size of dose per fraction is not increased and the interfraction interval is sufficient for repair to be completed). These concepts became a basic rationale for the development of various altered fractionation strategies as an alternative to conventional fractionation[13,14]. Simultaneously with reduced treatment time schedules, multiple fraction per day regimens have been used[15-17]. They allow a higher total dose to be given within the tolerance of late responding normal tissues.

Table 3 Grade of acute reaction (RTOG) of esophagus and bronchus according to fractionation regimen *n* (%)

Groups	Ⅰ	Ⅱ	Ⅲ	Ⅳ	*Z*	*P*
Esophagitis					0.855	0.376
CAIR	6 (20.7)	19 (65.5)	4 (13.8)	0		
CR	11 (37.9)	13 (44.8)	5 (17.2)	0		
Tracheitis					0.051	0.959
CAIR	14 (48.3)	10 (34.5)	5 (17.2)	0		
CR	13 (44.8)	13 (44.8)	3 (10.4)	0		

One modality currently in use to achieve this goal is the concomitant boost schedule designed by Maciejewski B *et al*[18] on carcinoma of the head-and-neck. Their data indicated when dose per fraction of 2.0 Gy given once-a-day at 24 h intervals, an analysis of severe mucosal reactions shows significant difference between CAIR group and CR group. Developed severe mucositis was 48% of patients and 5%, respectively. Their conclusion was that the accelerated treatment, using daily fractions of 2.0 Gy, 7 d per week, gives unacceptable toxicity. When dose per fraction was lowered from 2.0 Gy to 1.8 Gy, the overall rate of acute mucosal reactions decreased to 10% as reported by Skladowski K *et al*[9]. Both the 3 years local control rate and the 3 years survival rate was improved.The 3-year local tumor control was 82% in the CAIR group and 37% in the CR group, and 3 years overall survival was 78% and 32%, respectively.

In our trial, dose per fraction was 2.0 Gy in both groups. The data showed that acute radiation esophagitis and tracheitis in both groups was mainly grade I-II, and the difference between the two groups was not statistically significant. No patient received treatment resulting in acute radiation reactions that could not be tolerated in in either group. The immediate response of two group patients to RT was statistically significant. The efficiency rate (CR plus PR) was 82.8% (24/29) and 58.6% (17/29), respectively, the CAIR group was significantly better than the CR group ($P = 0.047$). The overall treatment time has been shortened by two weeks, as given in the result section.

The present trial demonstrates that seven-day-per-week continuous accelerated irradiation provides significant therapeutic benefit for patients with esophageal carcinoma with regard to both response and toxicity to RT. Fractions of 2.0 Gy could keep acute radiation esophagitis and tracheitis on a tolerable level (different from Maciejewski B). The results of our trial suggest that local control in patients with esophageal carcinoma might be improved by CAIR compared to CR methods, when using dose escalation by continuous accelerated irradiation (with sufficiently long interfraction intervals) and a shorter overall treatment time.

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RAPID COMMUNICATION

Application of laparoscopy in diagnosis and treatment of massive small intestinal bleeding: Report of 22 cases

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Abstract

AIM: To investigate the diagnostic and therapeutic value of laparoscopy in patients with massive small intestinal bleeding.

METHODS: Twenty-two patients with massive small intestinal bleeding and hemodynamic alteration underwent laparoscopic laparotomy in our unit from December 2002 to April 2005. Post pathologic sites were found, laparoscopy- or laparoscopy-assisted part small intestinal resection including pathologic intestinal site and enteroanastomosis was performed in all these patients.

RESULTS: The bleeding sites were successfully detected by laparoscopy in all these 22 patients. Massive small intestinal bleeding was caused by jejunum benign stromal tumor in 8 cases, by jejunum potential malignant stromal tumor in 5 cases, by jejunum malignant stromal tumor in 1 case, by Mechel's diverticulum in 5 cases, by small intestinal vascular deformity in 2 cases, and by ectopic pancreas in 1 case. A total of 16 patients underwent laparoscopy-assisted enterectomy and enteroanastomosis of small intestine covering the diseased segment and 6 patients received enterectomy of the diseased segment under laparoscope. No surgical complications occurred and the outcome was satisfactory.

CONCLUSION: Laparoscopy in diagnosis and treatment of massive small intestinal bleeding is noninvasive with less pain, short recovery time and definite therapeutic efficacy.

Key words: Small intestine; Bleeding; Laparoscopy; Meckel's diverticulum; Stromal tumor

INTRODUCTION

Massive small intestinal bleeding is rare, accounting for 0.4% of all cases of gastrointestinal bleeding. So far there has been no effective method for its definite diagnosis due to its nonspecific clinical symptoms and signs, thus its treatment is a challenge for clinical surgeons[1-3]. From December 2002 to April 2005, 22 patients with suspected massive small intestinal bleeding admitted to our department received laparoscopy for definitive diagnosis and treatment. The outcomes were satisfactory.

MATERIALS AND METHODS

Clinical data

Twenty-two patients with suspected massive small intestinal bleeding (13 males and 9 females) were included in the present study. Their age ranged from 16 to 61 years with a mean age of 38.6 years. The course of the disease ranged from 20 d to 10 years with a mean of 36.6 mo. The patients had recurrent massive bleeding, ranging from 3 to 15 times prior to admission with a mean of 5 times. The chief complaints were bright red or catsup-like bloody stools with concurrent abdominal pain in 4 cases. On the day of admission, the amount of bleeding was more than 1200 mL with concurrent hemodynamic changes. Hemoglobin ranged from 40.6 g/L to 66 g/L with a mean of 50.6 g/L. Massive small intestinal bleeding was suspected after bleeding from gastric duodenum, colon and rectum was excluded. Double contrast of barium and air examination of the alimentary tract were performed in 6 patients, of them 1 had small intestinal tumor and 1 had Meckel's diverticulum. Small intestine enteroscopy was carried out in 5 patients, of them 2 had small intestinal bleeding due to Mechel's diverticulum and 1 had small intestinal stromal tumor (Figures 1A and B). Emission CT scanning (ECT) of the abdominal cavity was performed in 6 patients, of them 2 had a small intestinal bleeding due to Mechel's diverticulum and small intestinal tumor (Figure 2). Digital subtraction angiography (DSA) was conducted

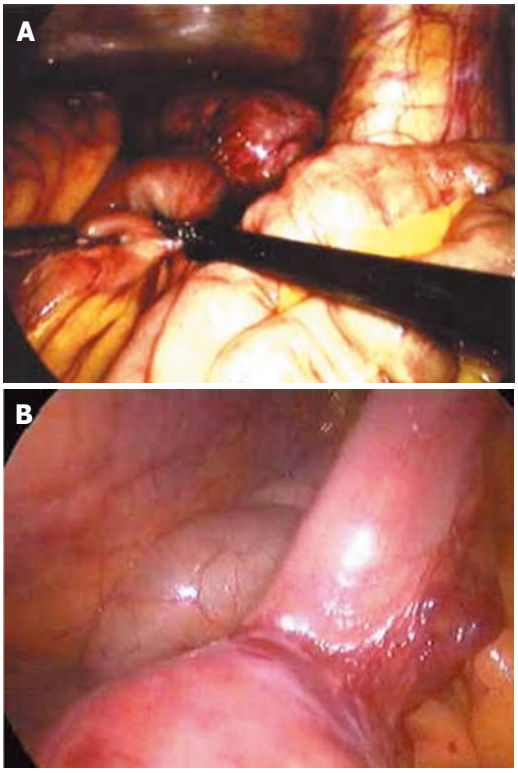

Figure 1 Laparoscopic laparotomy showing small intestinal bleeding (**A** and **B**).

in 3 patients, of them 1 had a jejunum tumor and 1 had the contrast medium in blood vessels of terminal ileum flowing into the intestinal cavity where pathological changes were not defined.

Surgical procedures

Before operation, shock was treated with blood transfusion until hemoglobin level reached 90 g/L or above. After the blood pressure became normal, emergency laparoscopic laparotomy was performed under general anesthesia with the patients at a head-down position. A transverse incision (1 cm) was made 0.5 cm inferior to the umbilicus to establish pneumoperitoneum with a pressure of 13 mmHg. A hole was made inferior to the umbilicus to insert the laparoscope of 10 mm at 30℃ to examine the abdominal viscera. Under the guidance of a laparoscope, a second and third holes of 5 mm were made at the level of umbilicus on the midlines of right and left clavicles. The second hole was used to check the whole small intestine from the part of the ileum and cecum to Treitz's ligament with a non-impairing laparoscopic bowel clamp, during which the proximal segment of intestinal tract from the boundary of the hematocele was observed and accumulated blood was squeezed out. The recurrent hematocele showed the definite bleeding site. When failing to reach the bleeding site, two alternative methods were used: perioperative small intestine enteroscopy to check the transparency of the intestinal wall with the help of a light source at the top of the enteroscope to localize the bleeding site, thus the small intestine near the hematocele was clamped out of the abdominal wall for incision and perioperative enteroscopy (Figure 3). After the bleeding site was localized, the third hole was dilated to make a 5 cm incision through the left rectus abdominis muscle to clamp the diseased intestinal segment, followed by resection of the diseased intestinal tract according to the laparotomy procedures (Figures 4A and B). In some cases, removal of the diseased small intestine and enteroanastomosis were performed under a laparoscope by transverse dilation of the hole inferior to the the umbilicus.

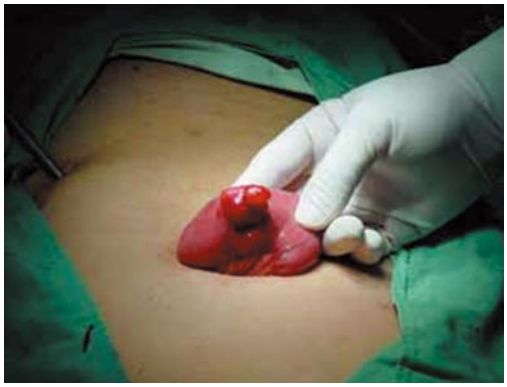

Figure 2 An oval stromal tumor causing small intestinal bleeding. Laparoscopic laparotomy found there is an oval stromal tumor on the up segment of jejunum, with clear borderline and smooth surface, having no adhesion with other tissue around it. Expansive abdominal excision was performed to draw out the tumor for resection.

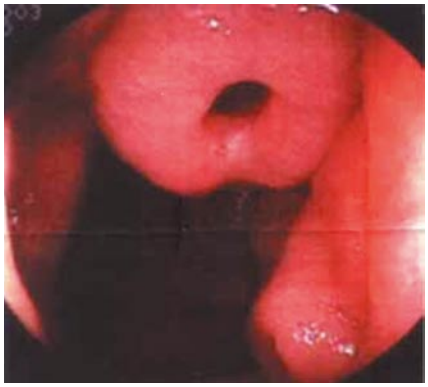

Figure 3 Electric intestinal endoscopy showing a benign jejunum tumor. A deep ulcer sunken on the top of the tumor could be seen.

RESULTS

The bleeding sites in these 22 patients were successfully found, of them 2 received laparoscopy combined with perioperative enteroscopy, 4 underwent perioperative incision of the small intestinal tract under laparoscope combined with perioperative enteroscopy under laparoscope. Massive small intestinal bleeding was caused by benign jejunum stromal tumor in 8 cases, by potential jejunum malignant stromal tumor in 5 cases, by malignant jejunum stromal tumor in 1 case, by Mechel's diverticulum in 5 cases, by small intestinal vascular deformity in 2 cases with 1 case having 2 sites of jejunum vascular dysplasia with concurrent intestinal mucous ulcer (the two sites were 10 cm and 40 cm from Treitz's ligament respectively) and by ectopic pancreas in 1 case. A total of 16 patients underwent laparoscopy-assisted enterectomy

and enteroanastomosis of small intestine covering the diseased segment and 6 patients received enterectomy to remove the diseased segment under laparoscope. The operative duration ranged from 45 min to 180 min with a mean of 90 min. After operation, all patients recovered passing flatus through the anus and taking food within 2 d. No surgical complications occurred and the mean postoperative hospitalization time was 6.5 d. Phone call follow-up was conducted for 2-24 mo with no recurrent alimentary tract bleeding. The surgical outcome was satisfactory.

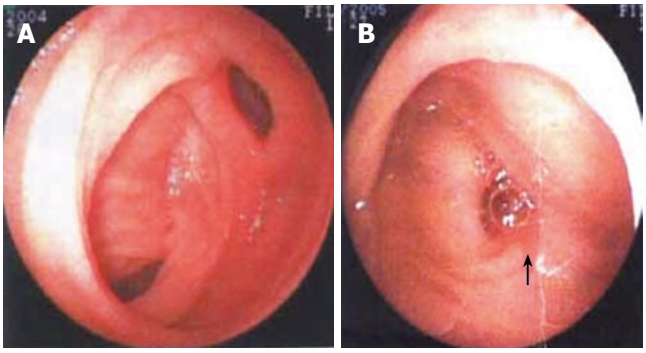

Figure 4 Double balloon enteroscopy showing the clamped diseased intestinal segment (**A**) and resected diseased intestinal tract (**B**). The double-balloon enteroscope was pushed 200 cm into the ileum through anus. Diverticulum was found in the ileum 90-100 cm away from the ileocecal valve, at the opening of which a 1.2 cm × 1.0 cm ulcer was observed. The ulcer had thin covering of lichenoid substance, but no active bleeding. No other abnormalities were found. Meckel's diverticulum was diagnosed.

DISCUSSION

The small intestine is about 3-5 m long, occupying three fourths of the whole gastrointestinal tract. The ansa intestinalis is circuitous overlapping active peristalsis and its location varies greatly in the abdominal cavity. Since massive small intestinal bleeding lacks specific clinical symptoms and signs, it is difficult to diagnose and locate it by routine examinations[4-6]. Small intestine enteroscopy is the most specific method for its diagnosis but its application is limited because this examination is time-consuming, extremely unpleasant, and causes bleeding and perforation with a high false positive rate[7-13]. Recently, a capsule endoscope is under clinic experiment, but it cannot perform biopsy and make pathological diagnosis[10-13]. Stromal tumor is the most frequent cause of small intestinal bleeding[1-3,5], Mechel's diverticulum and vascular conditions are the second frequent cause of small intestinal bleeding[1,4,6]. False positive tumor may not be shown on X-ray imaging of the whole alimentary tract because the bleeding foci often grows in exogenesis[14,15]. During the active stage of small intestinal bleeding, DSA can find the contrast medium flowing from the tumor site into the intestinal tract, showing local shadow with a slightly high density and embolism treatment can be conducted during diagnosis[15]. ^{99m}Tc-sestamibi is sensitive to mild intestinal bleeding , thus marking erythrocytes for gastrointestinal bleeding imaging, while it has no diagnostic value in the resting phase of bleeding or the bleeding being less than 0.05 mL/min[16]. At present, the diagnosis of massive obscure gastro-intestinal bleeding is usually made by exposure laparotomy, which is invasive with a false positive rate of 5%. Besides, patients with massive small intestinal bleeding are often weak with poor conditions and unstable vital signs, which prevent them from undergoing a major surgical operation.

Laparoscopy can clearly, directly and conveniently observe the whole intestinal serosa and mesentery and the small intestinal conditions can be managed with its assistance[11,16-20]. From December 2002, we have tried to use laparoscopy to manage obscure gastrointestinal bleeding in patients with massive small intestinal bleeding. The outcomes showed that laparoscopy could find the bleeding site of massive obscure gastro-intestinal bleeding. It is noninvasive with less pain and short recovery time. We believe that laparoscopy has a promising prospect in diagnosis and treatment of acute massive small intestinal bleeding and can be used as a routine method for the management of massive small intestinal bleeding[11,16,18,20]. Since intestinal stromal tumor and ectopic pancreas that cause small intestinal bleeding are generally small, examination followed by laparotomy cannot find the bleeding foci. Perioperative small intestine enteroscopy in combination with removal of hematocele can avoid the disadvantages of enteroscopy, such as time-consuming, extreme unpleasantness and complications of bleeding and perforation. Laparoscopy can find the bleeding foci, showing the advantages of noninvasive surgery. For those whose bleeding site is not defined by laparoscopy, perioperative enteroscopy of small intestine generally can reach the definite bleeding foci, deserving wide promotion[11,17,19,20].

In the present study, small intestinal bleeding occurred, leading to insufficient blood volume, the average hemoglobin level was 50.6 g/L. Once laparoscopy is accepted by patients, the laparoscope equipment and surgical appliances should be prepared as fast as possible for immediate surgery when shock takes a favorable return. The patient should lie on his/her left side at the head-down position. Firstly, parenchymatous viscera should be generally examined, followed by examination of the whole small intestine. This part of the ileum and cecum has a relatively stable location in the abdominal cavity and thus is easily exposed. Cecum should be used as the landmark during laparoscopic exploration, which starts from the terminal ileum with each 10 cm as one segment to the Treize's ligament. One patient had 2 sites with small intestinal vascular deformity so that exploration of the whole small intestine segment by segment was emphasized to avoid missing any focus. The laparoscopic exploration of small intestinal hemangiomas or vascular deformity should be more careful. The intestinal wall should be carefully explored for local prominence, pitting, overlapping and abnormal mesentery. The suspected bleeding segment should be palpated carefully with clamps to feel its hardness, flexibility, and activity. In case of active massive bleeding, intestinal peristalsis is active and the blood often accumulates in the distal bleeding segment which is dark blue under laparoscope. The suspected foci can be confirmed if emptied, blocked and reformed

hematocele is found. The time-consuming examination is mainly due to repeated enteroscopy. One patient with a history of 3-year bleeding had no positive laparoscopic findings. Repeated examinations had no other positive findings. A slightly hard intestinal wall of this part was touched during exploration, which was ectopic pancreas confirmed by pathological biopsy.

After the bleeding site was found by laparoscopy, laparoscopy-assisted enterectomy and enteroanastomosis were performed, during which an exploratory incision about 5 cm in length was made at the umbilicus level on the midline of the left clavicle to remove the diseased intestinal segment. The resected part of the small intestine should be 5 cm longer than the bleeding site that may result in a fast and reliable excision with light contaminations in the abdominal cavity. Enterectomy and enteroanastomosis can be performed under laparoscope in those whose bleeding sites are adjacent to the Treize's ligament, thus the diseased segment can be conveniently removed[1,2,11,16-20].

In conclusion, laparoscopy in diagnosis and treatment of massive small intestinal bleeding is noninvasive with less pain, short recovery time and definite therapeutic efficacy and has rather good clinical application prospects.

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CASE REPORT

Pravastatin: A potential cause for acute pancreatitis

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Abstract

Acute pancreatitis (AP) secondary to drugs is uncommon, with an incidence ranging from 0.3% to 2.0% of AP cases. Drug-induced AP due to statins is rare, and only 12 cases have thus far been reported. In this case report, we report a case of a 50-year-old female on pravastatin therapy for 3 d prior to developing symptoms of AP. The common etiological factors for AP were all excluded. The patient was admitted to the intensive care unit secondary to respiratory distress, though she subsequently improved and was discharged 14 d after admission. Although the incidence of drug-induced AP is low, clinicians should have a high index of suspicion for it in patients with AP due to an unknown etiology. Clinicians should be aware of the association of statins with AP. If a patient taking a statin develops abdominal pain, clinicians should consider the diagnosis of AP and conduct the appropriate laboratory and diagnostic evaluation if indicated.

Key words: Drug-induced pancreatitis; Acute pancreatitis; Statins; Pravastatin.

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INTRODUCTION

Acute pancreatitis (AP) secondary to drugs is uncommon, with an incidence ranging from 0.3% to 2.0% of AP cases[1]. The literature on drug-induced AP consists mostly of case reports, though there have been reviews analyzing the association of various drugs with AP[1-3]. The following drugs have been definitely associated with AP in many of the reviews: azathioprine, chlorothiazide, hydrochlorothiazide, estrogens, furosemide, sulfonamides, tetracycline, L-asparaginase, sulindac, valproic acid, didanosine, salicylates, aminosalicylates (mesalamine, sulfasalazine), calcium, sodium stibogluconate, pentamidine, vinka alkaloids, and metronidazole. There are many other drugs which have been implicated as having probable or possible associations with AP, including 6-mercaptopurine, methyldopa, ACE inhibitors, clozapine, rifampicin, cyclosporine, and many other drugs.

There have been 12 cases of AP associated with statins described thus far[4-14]. In four of the cases presented, reintroduction of the statin led to a recurrent attack of AP[4-6,12]. In this case report, we report a case of AP associated with pravastatin therapy. In view of the magnitude of use of statins in prevention of coronary artery disease, even an infrequent occurrence is worth reporting.

CASE REPORT

A 50-year-old female presented to our hospital with right upper quadrant abdominal pain, nausea, and vomiting for 1 d. Four days prior to admission, she was started on 10 mg pravastatin by mouth daily, though the patient stopped this medication the day prior to admission as she attributed her symptoms to the new medication. Her lipid panel one month prior to admission showed a total cholesterol level of 262 mg/dL, triglyceride level of 268 mg/dL, high-density lipoprotein (HDL) level of 52 mg/dL, and low-density lipoprotein (LDL) level of 156 mg/dL. She was also treated for hypertension with 10 mg enalapril by mouth daily for the past 18 mo, and 25 mg hydrochlorothiazide by mouth daily for 6 years. She took 2.5 mg olanzapine by mouth daily for the past year for severe anxiety, and a combination of 500 mg/1 mg metformin and rosiglitazone (Avandamet™) in the past year for type Ⅱ diabetes mellitus. She had osteoarthritis of both knees, and took 325 mg/37.5 mg one to two tablets of acetaminophen/tramadol (Ultracet™) as needed for pain. Of note, she was on atorvastatin 2 years prior to admission for a period of 3 d, though this was discontinued secondary to generalized body pain. Laboratory tests were not performed at that time. She had a cholecystectomy 16 years prior to admission. The common etiological factors for AP such as alcoholism, trauma to the abdomen, HIV disease, hypertriglyceridemia, and hypercalcemia were all excluded.

Table 1 Laboratory values

	Admission	48 h
Amylase (U/L)	914	280
Lipase (U/L)	1613	261
WBC (mm^3)	26300	21500
HCT (%)	53.9	42.4
LDH (IU/L)	389	645
Glucose (mg/dL)	495	
AST (IU/L)	18	
ALT (IU/L)	28	
Calcium (mg/dL)	10.2	6.5
PO_2 (mmHg)		76
BUN (mg/dL)		17
Base deficit		-1
Fluid sequestration (L)		2.8
Total cholesterol (mg/dL)	202	
Triglycerides (mg/dL)	118	

On physical examination, her blood pressure was 158/104 mmHg, heart rate was 131 beats per minute, respiratory rate was 30/min, temperature was 98 degrees Fahrenheit, and SpO_2 was 93% breathing room air. Her abdominal examination revealed hypoactive bowel sounds, with diffuse tenderness without rebound tenderness or guarding.

Laboratory values are included in Table 1. Three out of five Ranson criteria were fulfilled on admission, and two out of six criteria were fulfilled after forty-eight hours.

She was admitted to the intensive care unit, and given isotonic intravenous fluids and meperidine for pain control. CT scan of the abdomen showed inflammatory changes within the pancreas, though no discrete peripancreatic fluid collections were noted. Abdominal ultrasound did not show biliary ductal dilatation. Magnetic resonance cholangiopancreatography showed a normal pancreatico-biliary system. She developed respiratory distress, though this resolved after support with BIPAP and diuretic therapy. She improved significantly following this, and was discharged 14 d after admission.

Of note, she was restarted on enalapril during the admission and continued to take enalapril without any adverse effects. She was not restarted on pravachol or hydrochlorothiazide. Olanzapine and metformin/rosiglitazone were also restarted without any adverse effects. The patient was last seen 4 mo after discharge, and her hypertension and diabetes were well controlled on enalapril, metformin, rosiglitazone, atenolol, and glipizide. She also continued to take olanzapine without any adverse effects.

DISCUSSION

Statin-induced AP is rare and only 12 cases have thus far been reported in the literature[4-14]. Although AP is a rare side effect of statin therapy, there seems to be a strong association between statins and AP, as there have been four cases where reintroduction of the statin has led to a recurrence of AP[4-6,12]. In our case report, the patient was on pravastatin for three days prior to symptom onset, and the outcome was favorable. A rechallenge test involving documenting AP development during treatment with a drug, its disappearance after stopping the drug, and recurrence after reintroduction of the drug, was not performed in this patient due to ethical issues. This would be the most reliable evidence that pravastatin caused AP in this patient.

No data about a potential mechanism for statin-induced AP are available at this time. In previously published cases of statin-induced pancreatitis, the duration of statin treatment until the onset of AP varied from 8 h to 7 years, though the vast majority of patients presented within 6 mo of introduction of the statin[4-14]. Generally, the outcome is favorable in statin-induced AP, though there was a fatality in one case after a four-month hospital stay[13].

A number of medications that our patient took are known to be associated with AP. Thiazide diuretics[15], ACE inhibitors[16], atypical antipsychotics[17], biguanides[18], and acetaminophen[19] have been associated with AP. However, continuation of all of the above medications, with the exception of the thiazide diuretic, did not precipitate AP.

In conclusion, though the incidence of drug-induced AP is low, clinicians should have a high index of suspicion for it in patients with AP due to an unknown etiology. A diligent review of medications should be performed, focusing on drugs that have been associated with drug-induced AP[1-3]. Clinicians should be aware of the association of statins with AP. If a patient taking a statin develops abdominal pain, clinicians should consider the diagnosis of AP and conduct the appropriate laboratory and diagnostic evaluation if indicated.

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CASE REPORT

Carcinoid of the ampulla of Vater: Morphologic features and clinical implications

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Abstract

Carcinoids involving the ampulla of Vater are rare lesions that may produce painless jaundice. The published data indicate that these tumors, in contrast to their midgut counterparts, metastasize in approximately half of cases irrespective of primary tumor size. Therefore, radical excision in the form of pancreaticoduodenectomy is recommended regardless of tumor size. As with other gastrointestinal carcinoid tumors, biological treatment with octreotide analogues can be applied to symptomatic patients. Tumor-targeted radioactive therapy is a newly emerging treatment option. We here report case of a carcinoid tumor of the ampulla of Vater presenting as painless jaundice in a 65-year old man and review the relevant literature, giving special attention to the morphologic features, clinical characteristics, and treatment modalities associated with this disease process.

Key words: Ampulla of Vater; Histopathologic features; Clinical characteristics; Radical excision

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INTRODUCTION

Carcinoid tumors belong to the family of neuroendocrine tumors, which usually grow slowly with distinct biological and clinical characteristics. The incidence of these tumors is approximately 2.5 in 100 000 people per year. The appendix is the most common location, accounting for roughly 60% of all cases, followed by the ileum, the rectum and the stomach. Approximately 2% of cases involve the duodenum, 1% the biliary tract, and 0.6% the pancreas[1]. Carcinoid tumors that involve the ampulla are an extremely rare clinical entity, with 93 reported cases[2-7]. We report another case of a carcinoid tumor of the ampulla of Vater presenting as painless jaundice. The relevant literature is reviewed, giving special attention to the morphologic features, clinical characteristics, and treatment modalities associated with this disease process.

CASE REPORT

A 65-year old otherwise healthy man presented with a 2-wk history of intractable pruritus. Review of systems was notable for dark urine. He denied clay-colored stool, diarrhea, flushing or dyspnea. Physical examination was remarkable for mildly icteric sclerae. There were no palpable abdominal masses, adenopathy, or café-au-lait spots. Laboratory studies indicated a markedly elevated level of alkaline phosphatase (712 U/L). Total bilirubin was 29 mg/L. Abdominal computed tomography (CT) scan revealed significant dilatation of both the common bile duct (CBD) and the pancreatic duct, without any obvious periampullary mass, retroperitoneal adenopathy or liver lesions (Figure 1).

Endoscopic retrograde cholangiopancreatography (ERCP) showed a prominent major ampulla with normal overlying mucosa. The CBD measured 17 mm with an abrupt "shoulder" in the region of the ampulla (Figure 2). The ampullary obstruction was relieved with temporary biliary stent placement. Subsequent endoscopic ultrasonography (EUS) identified a 23 mm × 27 mm well circumscribed, round, hypoechoic mass in the region of the ampulla adjacent to the biliary stent, which was distinct from the pancreatic tissue and the duodenal wall (Figure 3). EUS-guided fine needle biopsy was suggestive of a carcinoid tumor. Positron emission tomography (PET) scan confirmed a lesion of increased metabolism in the vicinity of the pancreatic head, and excluded distant metastases. Urinary 5-hydroxyindoleacetic acid (5-HIAA), and serum carcinoembryonic antigen (CEA), CA19-9, serotonin, somatostatin and chromogranin A levels were all within normal limits.

A pylorus-preserving pancreaticoduodenectomy was performed. Pathology revealed a T_3, N1 ampullary carcinoid extending into the peripancreatic fat, with 7/13 positive lymphnodes. Immunohistochemically, tumor cells expressed chromogranin A and synaptophysin, and focally serotonin and somatostatin, which were stained negative

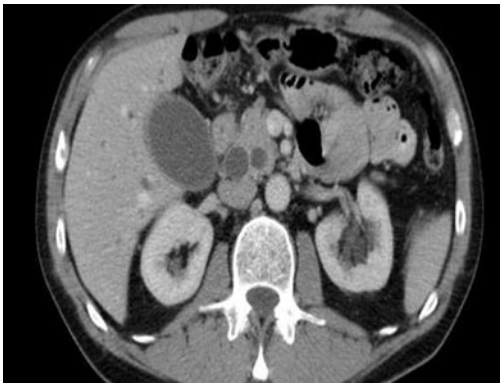

Figure 1 The characteristic "double duct sign" on abdominal CT is indicative of obstruction at the level of the ampulla.

for insulin, glucagon, and gastrin. The patient recovered uneventfully and was discharged to home on the seventh postoperative day. The follow-up plan consists of structural studies and biochemical markers every 3 mo up to 1 year and every 6 mo thereafter. At 9 mo he remained asymptomatic with no radiographically or biochemically detectable disease.

DISCUSSION

In 1888 the first case of a carcinoid tumor was reported as a lesion of the ileum[8]. Oberndorfer coined the term *karzinoide* ("carcinoma-like") in 1907 to distinguish these presumptively benign neoplasms from the malignant cecal adenocarcinomas[9]. Carcinoids have been detected in the entire gastrointestinal tract, from the esophagus to the rectum, as well as in extra-gastrointestinal locations, such as the bronchus, the testis, the ovary and the larynx. In contrast to the relative rarity of duodenal carcinoids, those of the ampulla of Vater represent a medical curiosity with only 93 cases reported in the world literature.

Indicative of their location, ampullary carcinoids cause jaundice as the leading symptom in approximately two-thirds of cases. Forty percent of patients present with abdominal pain and few with pancreatitis, weight loss, or malaise[2-7]. Every fourth patient has von Recklinghausen's disease[2]. The etiology is not clear, but it is likely that mutation of the NF-1 tumor suppressor gene may predispose to the development of ampullary carcinoids. In contrast to midgut carcinoids, foregut carcinoids are very rarely associated with carcinoid syndrome. In the largest reported series of 73 ampullary carcinoid cases, a history of flushing, diarrhea, or asthma was found in only 2 patients who also had extensive liver metastases[2]. Walton *et al*[4] have described a "variant" form of carcinoid syndrome that may occur in association with gastric or other foregut carcinoid tumors, which may be hidden by the elaboration of other hormonally active substances, such as adrenocorticotropic hormone, parathyroid hormone, calcitonin, gastrin, vasoactive intestinal peptide, growth hormone, or insulin, but responds to treatment with antihistamines.

Immunohistochemistry is of principal importance in accurately diagnosing ampullary carcinoids. Makhlouf *et al*[7] compared the immunohistochemical features of 12 ampullary and 53 duodenal carcinoids and demonstrated that the former group always expresses chromogranin A, but almost never expresses gastrin which has been identified in 56% of cases of duodenal carcinoids. Apart from its role in immunohistochemical diagnosis, chromogranin A has proven particularly useful as a tumor marker for monitoring disease response and progression in patients with gastrointestinal carcinoids. Analyzing data from 301 such patients, Janson *et al*[10] found that serum chromogranin A level > 5000 μg/L (normal value: 0-76 μg/L) is an independent predictor of poor survival.

Surprisingly, in the case of ampullary carcinoids, tumor size does not correlate with metastatic potential. In the case review by Hatzitheoklitos *et al*[2], metastasis is present in 46% of ampullary carcinoids > 2 cm, in 50% of tumors between 1-2 cm, and in 66% of tumors < 1 cm. Makhlouf *et al*[7] have reported two tumors measuring less than 2 cm demonstrating metastases, as well as a 5 cm tumor without any evidence of metastatic disease. These data indicate that carcinoids involving the ampulla of Vater metastasize in approximately half of the cases regardless of tumor size.

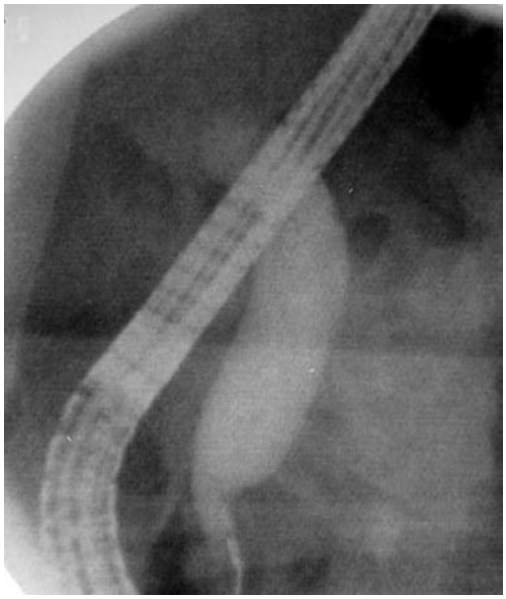

Figure 2 ERCP showing a markedly dilated CBD with an abrupt "shoulder" in the region of the ampulla, suggestive of a periampullary mass.

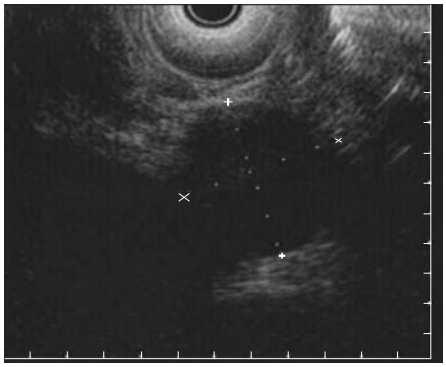

Figure 3 EUS identifying a 23 mm x 27 mm well circumscribed, round, hypoechoic mass in the region of the ampulla, which is distinct from the duodenal wall.

This is opposed to the classic teaching regarding midgut and hindgut carcinoids, in which the incidence of metastasis is felt to be a function of tumor size and is significantly higher with larger tumors.

Accordingly, the size of ampullary carcinoids cannot predict node-positive status, and therefore cannot determine the extent of operation. Should every patient with this tumor undergo a pancreaticoduodenectomy? The small number of cases and reported follow-up in the literature are not sufficient to answer this question definitively. Instead, each case should be individualized realizing that the goal is complete tumor removal. Given the propensity of ampullary carcinoids smaller than 2 cm to show nodal involvement, and considering the safety of pancreaticoduodenectomy in experienced hands, radical excision should be the treatment of choice to completely extirpate the tumor-bearing tissue. It should be observed that in multiple reported cases, long-term survival has been achieved by local excision of the ampulla[2]. These recommendations, however, were made during a period of surgical history when the operative mortality rate for pancreaticoduodenectomy was high, and therefore are not applied currently.

In cases of liver metastases, surgical resection or other cytoreductive techniques, such as radiofrequency ablation and chemoembolization, have been shown to improve hormone-mediated symptoms, quality of life and survival in certain groups of patients[11]. Patients with slowly growing carcinoid tumors do not generally benefit from cytotoxic chemotherapy. Somatostatin analogues can induce a symptomatic and biochemical response, but more recent studies have also indicated a cytostatic effect[11]. Tumor-targeted radioactive treatment with ^{90}yttrium and ^{177}lutetium coupled to a somatostatin analogue is currently under clinical evaluation[12]. Preliminary data indicate interesting clinical potentials.

In summary, carcinoids of the ampulla of Vater are rare tumors. They can cause symptoms mainly secondary to their periampullary location. Up to 25% of patients have von Recklinghausen's disease. Carcinoid syndrome is uncommon, unless hepatic metastasis is present. Chromogranin A is an important tumor marker. Determination of histopathology is of utmost importance and involves specific immunohistochemical staining. Aggressive operative extirpation is the cornerstone of treatment and provides the only chance for cure. Biological treatment with somatostatin analogues can be applied in symptomatic patients with slowly growing neoplasms. Tumor-targeted radiotherapy has been introduced recently with promising results. Future therapy will be based on specific tumor biology and treatment will be customized for each individual patient.

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CASE REPORT

Retroperitoneal fibrosis: A rare cause of both ureteral and small bowel obstruction

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Abstract

Retroperitoneal fibrosis (RPF) is a rare condition of unclear etiology. It can cause ureteral obstruction. We present the unique case of a 54 years old female, who initially presented with spontaneous perforation of the cecum. Upon exploring the abdomen, the classical glistening white, unyielding retroperitoneal fibrosis was encountered. A right hemicolectomy was performed. Subsequently, the patient presented with bilateral ureteral obstruction, and later on with small bowel obstruction. Ureteral obstruction was treated with stents, and small bowel obstruction was treated with bypass. To our knowledge no case of idiopathic RPF presenting with features of both bilateral ureteral and small bowel obstruction has been reported in the literature.

Key words: Retroperitoneal fibrosis; Ureteral obstruction; Small bowel obstruction; Surgery for retroperitoneal fibrosis

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INTRODUCTION

Retroperitoneal fibrosis (RPF) is a rare condition of unclear etiology. It is believed to be immune-related. About two-thirds of the cases are thought to be idiopathic. We present a case of idiopathic RPF in a 54 years old female who developed bilateral hydronephrosis and then small bowel obstruction due to extrinsic compression from retroperitoneal fibrosis.

To our knowledge no case of idiopathic RPF presenting with features of both bilateral ureteral and small bowel obstruction has been reported in the literature. We recommend early surgical intervention in patients with advanced RPF who are unresponsive to steroids.

CASE REPORT

A fifty four years old African-American female came to the emergency room with a five-day history of abdominal pain. She described it to be sharp in nature and localized mostly in the middle of the abdomen. She graded pain to be seven on a scale of zero to ten. The pain had no radiation or shifting. The patient also complained of nausea and three episodes of vomiting which was bilious in nature. The patient passed her last bowel movement three days ago and it was normal. She denied fever or shaking chills. Her past medical history was significant for hypertension, type Ⅱ diabetes mellitus and asthma.

Her past surgical history was significant for laparoscopic cholecystectomy done 6 years ago. On physical examination, she was afebrile and her vital signs were stable. She was awake and alert. She had normal heart sounds, and bilateral breath sounds. Her abdomen was soft, and had generalized and reboud tenderness. Her white blood cell count was 18000 cells/mm^3; the hemoglobin was 14 mg/dL and platelet count was 253 cells/mm^3. Her serum chemistry and coagulation tests were normal. Her flat and upright abdominal radiographs were normal. CT of the abdomen and pelvis showed extravasation of oral contrast and inflammation around the ascending colon (Figure 1). She was brought to the operating room and an exploratory laparotomy was performed via a midline incision. On entering the peritoneal cavity, dense intra-abdominal adhesions were encountered at the hepatic flexure. The classical glistening white, unyielding retroperitoneal fibrosis was encountered. The plaque predominantly encased both kidneys. It extended over the renal pelvis and upper ureters on both sides up to the level of aortic bifurcation and encased the great vessels in the midline. A 1 cm × 2 cm perforation was found in the cecum. A right hemicolectomy was performed. Pathology report showed fibrinous adhesions and inflammatory reaction around the site of perforation. No evidence of any tumor was seen. Patient's post-operative course was uneventful. She started a clear liquid diet on post-operative d 2, and was gradually advanced to a regular diet. The

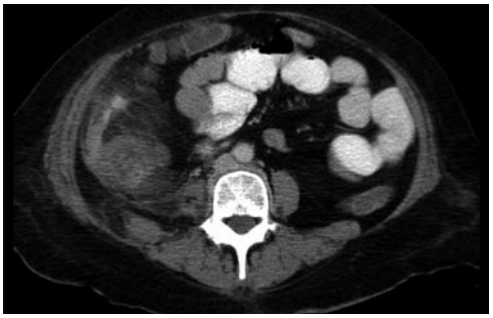

Figure 1 CT of Abdomen showing inflammation around right colon and extravasation of oral contrast.

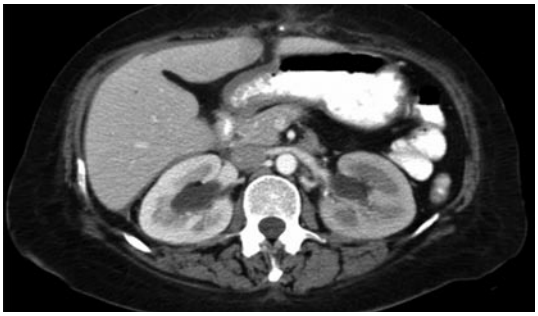

Figure 2 CT of abdomen showing bilateral hydronephrosis.

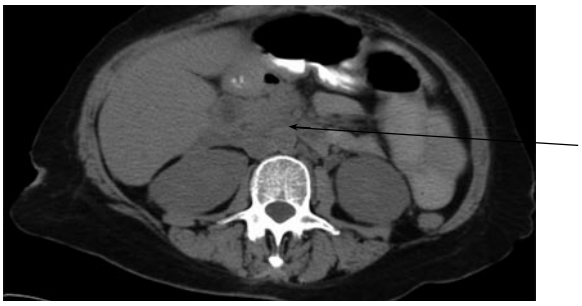

Figure 3 CT of abdomen showing retroperitoneal fibrosis, encasing aorta and inferior vena cava.

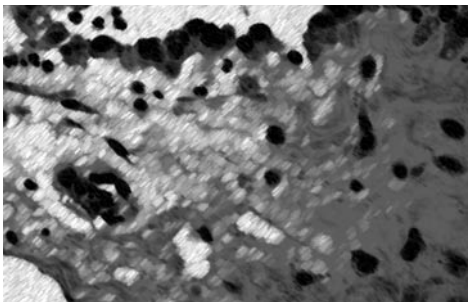

Figure 4 Hematoxylin and Eosin Staining showing fibrinous adhesions and inflammatory reaction.

patient was diagnosed with retroperitoneal fibrosis and, hence, received oral steroids. The patient was discharged home on post-operative d 6. She was examined in clinic 4 wk after surgery and reported no complaints.

Three months after surgery, the patient presented to the emergency room with complaints of severe back pain. Her examination was normal. Her laboratory tests were normal. CT scan of the abdomen showed marked hydronephrosis of the right kidney. She underwent cystoscopy which showed narrowing of the right proximal ureter. A stent was placed in the right ureter. The stent was removed 3 wk after placement.

She presented to emergency room after 9 mo with complaints of back pain. Her physical examination and laboratory work was normal. CT of abdomen and pelvis showed bilateral hydronephrosis (Figure 2). A cystoscopy was performed which showed a normal bladder. Retrograde pyelogram showed normal ureters until the proximal part of both ureters, where tortuosity and dilation of the renal pelvis on both sides was noted. Bilateral ureteral stents were placed.

Eight months after bilateral stent placement, she presented to the hospital with complaints of severe abdominal pain. The pain was localized to the middle of the abdomen. It was sharp in intensity and had no radiation and no shifting. The pain was associated with repeated bouts of vomiting. On physical examination, she was afebrile and her vital signs were stable. She was awake and alert. Her abdomen was soft and not distended. She had tenderness in the right upper quadrant. Her white blood cell count was 34000 cells/mm^3, and the hemoglobin was 13 mg/dL. All other laboratory values were in the normal range. Abdominal radiographs showed multiple air fluid levels. CT of her abdomen showed multiple dilated loops of the small bowel and thickening of the bowel wall. Transition point appeared to be somewhere between the jejunum and the ileum. The colon was normal (Figure 3). An exploratory laparotomy was performed through midline incision. On opening the abdomen, the findings were that of severe fibrotic reaction with almost a frozen abdomen on the right lower aspect. Once again, classical glistening white, unyielding retroperitoneal fibrosis was encountered. Careful lysis of adhesions was done and the left upper quadrant was explored, whereby dilated loops of small bowel were found. Lysis of adhesions was attempted in the right lower quadrant but was encountered with enormous amount of bleeding. It was felt that the obstruction was caused by the external compression from retroperitoneal fibrosis. A decision was made to bypass the dilated segment of the jejunum into the colon without interfering with the frozen abdomen on the right side. The ligament of Treitz was identified and the dilated small segment was seen up to about 200 cm of the jejunum. The last portion of jejunum which entered into the frozen part of the abdomen was identified. A 10 cm long segment was anastomosed with the transverse colon which was free of adhesions. Side-to-side anastomosis was done using a GIA-75 and a TA60. Irrigation of the abdomen was carried out. The abdominal cavity was closed. The postoperative course was uneventful. On postoperative d 2, the patient's nasogastric tube was discontinued. A clear liquid diet was started on postoperative d 3 and was gradually replaced with regular diet. The patient was discharged home on post operative d 9. Pathology report showed fibrinous adhesions and inflammatory reaction (Figure 4).

DISCUSSION

Classical idiopathic retroperitoneal fibrosis, also known as Ormond's disease, was described in 1948[1]. The typical retroperitoneal fibrosis is a fibrotic process encasing the abdominal vessels and the ureter with an epicenter at the level of L4-5[2]. RPF has an inciting agent in one-third of the cases, while the remaining two-thirds have no known cause[2].

CT scan of the abdomen is the imaging investigation of choice. Cases with ureteric obstruction can be successfully treated with steroids alone[3], and occasionally with tamoxifen[4], but ureterolysis may still be necessary in advanced or unresponsive cases. This case is unusual because the patient did not respond to oral steroids and had symptomatic ureteral and small bowel obstruction due to retroperitoneal fibrosis. We recommend early surgical intervention in patients with advanced RPF who are unresponsive to steroids.

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American College of Gastroenterology
Annual Scientific
20-25 October 2006
Las Vegas, NV

14th United European Gastroenterology Week, UEGW
21-25 October 2006
Berlin, Germany

APDW 2006: Asian Pacific Digestive Week 2006
26-29 November 2006
Lahug Cebu City, Philippines

EVENTS AND MEETINGS IN THE UPCOMING 6 MONTHS

Falk Symposium 151: Emerging Issues in Inflammatory Bowel Diseases
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10th International Congress of Obesity
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Easl 2006 - the 41st annual
26-30 April 2006
Vienna, Austria
Kenes International

Prague hepatology 2006
14-16 September 2006
Prague
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Falk Symposium 152: Intestinal Disease Part I, Endoscopy 2006 - Update and Live Demonstration
4-5 May 2006
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Falk Symposium 153: Intestinal Disease Part II, Immunoregulation in Inflammatory Bowel Disease - Current Understanding and Innovation
6-7 May 2006
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3-6 May 2006
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5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764]

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

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Books

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

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